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The Cytotoxic and Endocrine-Disrupting Potential of Alcohol Ethoxy Sulfates in *Oreochromis niloticus* Adrenocortical Steroidogenic Cells Exposed In Vitro

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Abstract

The cytotoxic and endocrine-disrupting potential of surfactant alcohol ethoxy sulfates in a common cichlid *Oreochromis niloticus* was investigated. The concentration ranges of AES in the field (1.00, 1.50, 2.00, and 2.50) mg/L, including the control, were exposed to the fish for 30 days. Glutathione-s-transferase (GST) and Reduced Glutathione (GSH) and cortisol levels were measured using a UV–VIS Spectrophotometer. There was no significant difference ($p > 0.05$) in the physiochemical parameters between the experimental group and the control. The effects of the surfactant on GST activity on day 2 of the exposure was slightly affected but was not significant ($P>0.5$), while days 9 to 16 witnessed moderate alteration that was significant at ($P< 0.05$) at the concentration of 2.0 and 2.5mg/L only. The changes were obvious on days 23rd and 30th in all the treatments and were highly significant ($p<0.01$). GSH activity in the blood serum of the investigated fish exposed to different concentrations of AES was not significantly ($p > 0.05$) affected. However, the enzyme activity was slightly higher than the control in all the treatments. In the control fish, there was a gradual increase in the cortisol level from day 2 (5.20) ug/dl to day 30 (5.30 + 0.010) ug/dl. In the treated fish, the increment in the cortisol level was proportional to the exposure concentration and was significant ($p < 0.05$) between the control and various treatments. Detergents seep into groundwater and enter rivers, harming fish and, indirectly, humans. By choosing the safest hazardous products, consumers can reduce chemical pollution

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Introduction

Cytotoxicity testing is fundamentally used in the production of a wide range of items, from pharmaceuticals to cosmetics, and also involves toxicity tests on plant products (which will be utilized for extracts and other applications). The term "selectivity index" is a measure of how selective a procedure is, and it describes the relationship between the potential biological activity of a plant specimen and the potential cytotoxicity of the plant specimen.

The endocrine system is a network of glands in the body that produces hormones that aid in the exchange of messages between individuals. Their work is directly related to the functioning of nearly every cell, organ, and function in the body (Predieri *et al.*, 2022). A consistent internal environment is maintained by the endocrine system, which promotes adequate communication between the body's many organs and tissues. An important function of the endocrine system is the body's ability to respond to and correctly cope with changes in the internal or external environment, such as those caused by stress or injury. When the endocrine system works to maintain homeostasis in the body, it is assisted by its communication with other systems such as the neurological system, the immune system, and the body's circadian mechanism.

Chronic exposure to a high number of chemicals affects communication between the neurological, endocrine, and immunological systems, resulting in hormonal imbalances that have deep and serious effects on both the physiological and behavioral levels of the individual (Rachdao and Sarkar, 2013).

Alcohol ethoxylate (AEs) is a type of surfactant that is found in products such as laundry detergents, surface cleaners, cosmetics, agricultural products, textiles, and paint (Federle *et al.*, 2002). In cosmetics and other commercial products, they can be composed wholly of linear alkyl chains or a mixture of both linear and mono-branched alkyl chains. A majority of the surfactants are released down the drain, where they may be adsorbed into particles and biodegrade through anaerobic processes, with 28–58 percent of them degrading in the sewer system (Jackson *et al.*,2016).

AEs will decay in the environment through aerobic and anaerobic processes, or they will be absorbed by plants and animals. Alcohol or alcohol ethoxylate, as well as ethylene glycol sulfate, are produced during the degradation of AES. The degradation process of AES is demonstrated by the formation of alcohol or alcohol ethoxylate, as well as the formation of ethylene glycol sulfate during the degradation process of AES (Rachdao and Sarkar, 2013).

The surfactant is not mutagenic, carcinogenic, or skin sensitizer, nor have they been found to have any impact on reproduction or development, however, one of its byproducts; ethoxylation 1,4-dioxane, has been linked to cancer in humans (Barbara and Jabeen, 2021). Similarly, undiluted AEs can cause skin or ocular irritation when applied topically individual (Stickney and Julie, 2003).

In the last 50 years, there has been a remarkable growth in the number of chemical substances used around the world as plasticizers, insecticides, detergents, paints, metals, food cans, flame retardants, cosmetics, and chemical waste, among other applications. These compounds can interfere with the endocrine systems of both humans and animals, according to research. It has been discovered that many different natural plant products share the same characteristics (Phyto-oestrogens). A comprehensive inquiry into the risks these drugs pose to public health has been launched in recent years as a result of legitimate worries about the dangers these compounds pose to human hormone balance.

The vast majority of surfactant ecotoxicity studies that are currently available use nominal concentrations rather than measured concentrations (likely the result of technical difficulties associated with analytical measurements of components of these surfactants). As a result, it could be argued that there is a limited degree of reliability in many current ecotoxicology studies.

Surfactants are very toxic and hazardous substances for aquatic organisms, and their everyday use in domestic and industrial fields encourages quantitative and qualitative examination of their effects on aquatic resources.

Materials and Methods

Pre-Analytical Stage

Fish experiments were carried out following all applicable rules and regulations. A greenhouse was built to simulate the fish's natural habitat, and it was cleaned daily. Mini ponds measuring 270 1/2 x 24 1/4 x 29 12 inches were built with clayey loam soil. The water in the ponds was kept at the following physicochemical parameters: 27.50 0.25oC, pH 7.2 0.03, dissolved oxygen 7.20 0.10 mg/l, total alkalinity 148 2.1 mg/l as CaCO3, and hardness 112 1.5 mg/l as CaCO3.

The *O.niloticus* used in this study were monitored from the egg stage until they reached the desired stage of maturation. The fish were fed protein and vitamin-rich fodder twice daily.

The fish produced by this setup was allowed to grow for 20 weeks to reach the desired size for toxicological testing

Toxicity Test

The fish were carefully delivered to the lab and acclimated to the environmental condition. The fish were moved to 10-liter plastic tubs after acclimatization. Each tub contained ten fish, and they were divided into five groups and kept at room temperature. The concentration ranges of Alcohol Ethoxy Sulfates reported in the field (1.00, 1.50, 2.00, and 2.50) mg/L, including the control, were exposed to the fish for 30 days. Fish in the control and experimental groups received twice-daily meals at 3% of their body weight throughout the experiment**.**

Every 24 hours, the water and toxicants were completely replaced, and the plastic tubs were kept as pristine as possible. Daily measurements of the water's physicochemical characteristics were taken throughout the investigation. After each experimental period (2, 9, 16, 23, and 30th), a fish was taken out of each plastic tub and its heart was punctured to collect blood samples. A sample of blood was drawn and put in labeled sample heparinized bottles for testing

Glutathione-s-transferase Determination

Blood was collected in a heparin vial and centrifuged at 4 °C for 10 minutes at 3,000 rpm. The top yellow plasma layer was gently pipetted into a vial without disrupting the white buffy layer and kept on ice – 80 °C – until analysis.

The total GST activity in the fish plasma was accessed using the Assay Kit. The Assay Kit uses CDNB, which is suitable for the widest spectrum of GST isozymes. GST catalyzes the conjugation of L-glutathione to CDNB by using the glutathione's thiol group.

GSH + CDNB $\longrightarrow$ GS-DNB Conjugate + HCl

The reaction product, GS-DNB Conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample

Determination of Reduced Glutathione (GSH): In many species, glutathione (GSH), a thiol-containing tripeptide (-glutamyl-cysteinyl-glycine), is an important antioxidant. It has been linked to the detoxification/elimination of xenobiotics and the preservation of protein sulfhydryl group oxidation states. GSH is also involved in the pathogenesis of a

variety of human disorders, including cancer and cardiovascular disease. Glutathione is found in cells in both reduced (GSH) and oxidized (GSSG) forms, with GSH being the most abundant under normal physiological conditions. The amount of GSH was determined using a modified version of Koyuncu *et al.* (2017). GSH samples were detected using a microplate reader (Spectra max M5, USA) with excitation at 345 nm and emission at 425 nm as the reference. The results in serum were represented as nmol/ml.

Cortisol Determination

96-well plates were used to measure cortisol concentration in duplicate samples of plasma. The marker concentration was calculated using a standard curve that was run on each plate and was adjusted for dilution factor and sample volume (plasma). The lower limit of detection was 52.4 pg/mL, and inter-assay variability was 4.32 percent

Statistical Analysis: The statistical significance between the control and the various treatments were compared using a paired t-test. At $P \leq 0.05$, it was deemed significant. The analysis was conducted using GraphPad InStat (version 3.00, GraphPad InStat Software Inc. 200).

Results

Physiochemical Parameters: The Physiochemical Parameters of the test media were monitored daily, and the average was recorded at the end of the investigation. The was no significant difference between the experimental group and the control ($p>0.05$)

Table 1: Physiochemical parameters of the control and various concentrations of AES (mg/L)

Parameters	0.00 Mean ± SE	1.00 Mean ± SE	1.50 Mean ± SE	2.00 Mean ± SE	2.50 Mean ± SE
PH	7.05 ± 0.06 [a]	7.10 ± 0.10[a]	7.30 ± 0.40[a]	7.10± 0.50[a]	6.90± 0.10[a]
Temperature (^{O}C)	27.50 ±0.10 [a]	27.30± 0.31[a]	27.10± 0.01[a]	27. 50 ± 0.10[a]	27.10 ± 0.70[a]
Alkalinity (mg/l)	13.90±0.20[a]	13.20±0.10[a]	13.70±0.10[a]	13.20±0.70[a]	13.30±0.20[a]
Total hardness (mg/l)	26.10±0.30[a]	26.10±0.30[a]	28.20±0.50[a]	26.10±0.10[a]	26.90±0.10[a]
Dissolve O_2 (mg/l)	7.90±0.30[a]	8.20±0.10[a]	8.90±0.21[a]	8.20±0.50[a]	8.80±0.40[a]

Physiochemical parameters of the test media during sub-lethal exposure of *C.nigrodigitatus* to concentrations (mg/L) of AES after 30 days of exposure. The mean with the same superscript in the row is significantly different * ($p >0.05$).

Biochemical Responses

The effect of the surfactant on GST activity is shown in Figure 1. On day 2, of the exposure, there was a slight alteration in the GST activity in all the exposures but was not significant ($P>0.05$). day 9 to 16 witnessed moderate alteration that was significant ($P<0.05$) at the concentration of 2.0 and 2.5mg/L only. The changes were obvious on day 23rd and 30th and were highly significant ($p<0.01$) irrespective of the treatment (Figure 1).

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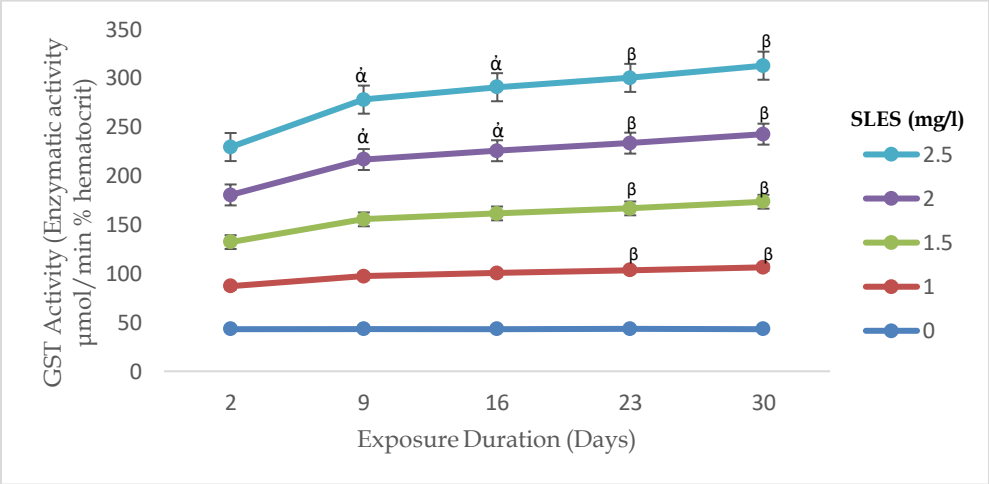

Figure 1: GST activities in the erythrocytes of *O. niloticus* exposed to sublethal concentrations of alcohol ethoxy sulfates; A symbol above bars indicates significant differences between the control and the experimental groups $^{\alpha}(p<0.05)$; $^{\beta}$ $(p < 0.01$

GSH activity in the blood serum of the investigated fish exposed to different concentrations of AES was not significantly ($p \geq 0.05$) affected. However, the enzyme activity was slightly higher than the control in all the treatments (Figure 2).

In the control, the enzyme activity ranges between 2.11 to 2.14 nmol/ml. In the treatments, there were fluctuations in the activity of the enzyme irrespective of the exposure durations but increased with SLES concentrations. The range at 1.0mg/l of AES on days 2 to 30 was 2.14 to 2.17 nmol/ml, at 1.50mg/l treatment, the range was 2.16 – 2.18 nmol/ml, at 2.00mg/l SLES, it was 2.16 – 2.18 nmol/ml. At the highest AES treatment of 2.50mg/l, the enzyme activity range was 2.16 – 2.50 nmol/ml.

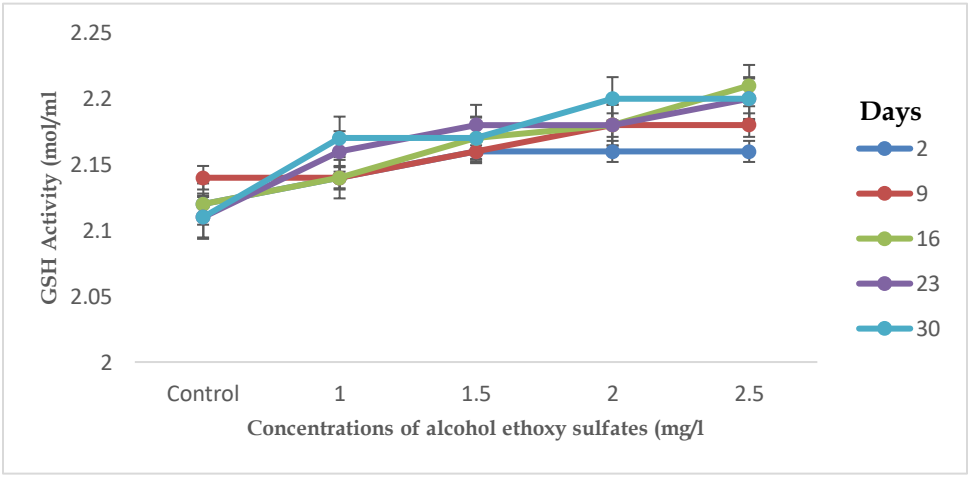

Figure 2: GSH activity in the serum of *o.niloticus* exposed to various concentrations of alcohol ethoxy sulfates; No significant difference ($p \geq 0.05$) between the control and treatments

In the control fish, there was a gradual increase in the plasma cortisol level from day 2 (5.20) ug/dl to day 30 (5.30 + 0.010) ug/dl. However, in the treated fishes, the increment was obvious as compared to the control fishes. On day 2 in the treated fishes, there was a spontaneous increase in the cortisol level with the increase in concentrations of AES on day 2, the concentration of 0.05mg/l the cortisol level was (6.1+ 0.010) ug/dl, and at 0.20ug/l concentration, the cortisol level was 7.20 + 1.03ug/l, and no significant ($p>0.05$)

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different between the control and various treatment on the same day. From day 19th to 30th, the increment in the cortisol level was proportional to the exposure concentration, and was significant ($p < 0.05$) between the control and various treatments (Table 2)

Table 2 : Responses of hydrocortisone (µg/dL) in the plasma of *O. niloticus* exposed to Low dose of Alcohol Ethoxy Sulfates (mg /L) .

Days	Control	1.00	1.50	2.00	2.50
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
2	5.20 ± 0.01[a]	6.10 ± 0.01 [a]	6.30 ± 0.15[a]	6.90 ± 0.20[a]	7.20 ± 0.18[a]
9	5.26 ± 0.01[a]	8.30 ± 0.10[b]	9.10 ± 0.50[b]	9.90 ± 0.12[b]	9.90 ± 0.80[b]
16	5.60 ± 0.08[a]	9.10 ± 0.10[b]	9.30 ± 0.20[c]	11.20 ± 0.50[c]	11.30 ± 0.20[c]
23	5.90 ± 0.02[a]	9.90 ± 0.20[b]	11.10 ± 0.10[c]	12.10 ± 0.10[c]	13.10 ± 0.10[c]
30	5.90 ± 0.01[a]	10.10 ± 0.13[b]	12.20 ± 0.20[c]	14.30 ± 0.20[c]	15.30 ± 0.90[c]

*: [a] not significant ($p \leq 0.05$); [b] significant ($p \leq 0.05$), [c] highly significant ($p \leq 0.01$)

Discussion

To identify the incidence and effects of xenobiotics, biochemical biomarkers are increasingly being used in ecological risk assessment of the ecosystem. This is due to their potential as a rapid early warning signal against potentially harmful stressor effects. Biochemical biomarkers, ideally, will detect effects at the subcellular level before they become visible at higher levels of subsidence.

We discovered stress-specific and time-dependent responses of fish at all biological entities studied in this study.

Glutathione is a vital detoxifying enzyme that aids in detoxification and the elimination of harmful toxins and pollutants. It protects against peroxidative damage and facilitates the conjugation of xenobiotics; glutathione-S transferase is secreted into the cell to protect it from free radicals. In this study, the activity of GST increased in the fish exposed to the toxicant in a concentration and time-dependent manner, with a statistically significant difference ($P < 0.05$).

Lipid peroxidase detoxification results in several byproducts, each of which, at larger quantities, has many detrimental biological effects., may be facilitated by GST-mediated conjugation. Induced GST activity demonstrated the role of this enzyme in xenobiotic toxicity resistance, and also suggested an induction of the hepatic detoxification process (Santos *et al*.,2004). Similarly, The amount of glutamate and glutamine in the cell determines how much glutathione is produced (DeBerardinis and Cheng, 2010).

The induction of GST activity observed in this study is in line with the findings from earlier investigations in fish exposed to different environmental stressors (Jin *et al*., 2010; Guilherme *et al*., 2012; Stara *et al*., 2012; Xing *et al*., 2012; Blahova *et al*., 2013; Nwani *et al*., 2013; Sinhorin *et al*., 2014)

GSH activity in the serum of the investigated fish was slightly increased in a concentration-dependent manner. The enzymes involved in the detoxification of xenobiotics and their metabolites are the biochemical indicators that have been highly investigated. According to Pereira *et al.* (2013), fish possess biotransformation enzymes that are primarily responsible for converting liposoluble substances into more readily excretable metabolites. By hydrolyzing the harmful molecules, this biotransformation process entails the Phase I detoxification process. The hazardous compounds can then be eliminated or continue along the biotransformation pathway (Di Giulio and Hinton, 2008). The conjugation of the metabolites generated in Phase I with the endogenous molecules of the cell occurs during the Phase II detoxification process. During the transformation of the hazardous substance, reactive oxygen species are formed, which can cause oxidative damage to cell structures (Rosa *et al.,* 2005). Oxidative stress is the result of an imbalance between the body's antioxidant defense system and the generation of free radicals that can peroxide lipid membranes in cells. Continuous enzymatic and non-enzymatic processes within the cell result in the generation of free radicals, which oxidize proteins, DNA,

and unsaturated lipids in the cell membranes. This results in the production of extremely unstable lipid hydroperoxides, whose byproducts are extremely reactive when they decompose, posing a threat to cell integrity (Shao *et al.*,2012) The endocrine response to pollutants is an integral part of the homeostatic physiological process activated in response to environmental stressors including pollutants. The hypothalamic-pituitary-adrenal (HPA) axis is crucial for the ability of vertebrates to cope with stressors. In fish, the end product of this axis (called the HPI axis in fish as they have interrenal cells in their head kidney rather than adrenals) is cortisol, which has both glucose and mineralocorticoid functions in these animals. But as in other vertebrates, the synthesis and release of cortisol by the interrenal cells in fish is controlled primarily by adrenoceptor-Cortisol is the most active and abundant corticosteroid in fish blood and its structure has been highly conserved in all of the vertebrate species in which it is found. The primary targets of cortisol action are the gills, intestine, and liver, which reflect the two main adaptive functions of cortisol identified to date: osmoregulation and the maintenance of a balanced energy metabolism.

Plasma cortisol is an excellent indicator of functional alterations in the HPI axis (Hontela, 2005).

Secretion of the steroid hormone cortisol by the interrenal tissue is a characteristic reaction of teleost fish to almost all forms of environmental stress. Exposure to metals and other toxicants that impair cortisol secretion could then influence social interactions and cortisol-dependent processes (Gagnon *et al.,* 2006). An elevation of plasma cortisol is the most widely used indicator of stress in fish. This may be considered as the reaction of the fish to recognize the presence of a noxious or potentially harmful substances in the environment. Scott *et al*. (2003) reported that plasma cortisol levels in rainbow trout increased when fish were exposed to an alarm substance, a chemical released from skin epithelium, and this increase was inhibited by cadmium. Hontela et al. (2006) observed that copper at high concentrations disrupts cortisol secretion through a direct toxic effect on adrenocortical cells while low concentrations resulting from a 30-day exposure to environmentally relevant Cu concentrations enhance cortisol secretion in response to ACTH in vitro.

Elevated cortisol level is probably related either to creating abnormal chloride and ATPase level or the process of trying to restore the values to normal, since corticoids have been implicated in electrolyte balance and gill ATPase activity (Fiess *et al.,* 2007).

The observed rise in plasma cortisol levels during the treatments in the present study may be attributed to various factors, such as the release of cortisol from the interrenal region as a stress response, abnormal plasma chloride levels, or the body's attempt to restore these values to their normal range (Gagnon *et al.,* 2006).

Conclusion

The contamination of the atmosphere, hydrosphere, or lithosphere by chemical substances resulting from anthropogenic actions is a significant cause of water pollution. Detergents, in particular, play a prominent role in this issue due to the presence of nitrates and phosphates within their composition. Laundry detergents, commonly referred to as surfactants, are chemical compounds employed to facilitate the cleansing process of garments, effectively eliminating soil particles and enhancing the lathering properties of soap. Exposure to this substance has the potential to exacerbate respiratory, ophthalmic, and cutaneous systems.

The contamination of groundwater, in addition to the runoff that enters lakes and rivers, presents a significant risk to the well-being of aquatic organisms and, consequently, human populations. The prevention of chemical contamination can be facilitated by individuals through the modification of their habits and lifestyles. Chemical waste reduction can be achieved by the adoption of strategies such as purchasing only the required quantities and giving priority to items with little potential for adverse effects. It is advisable to exclusively utilize the several phosphate-free detergents that are currently accessible.

Recent research has indicated that alcohol ethoxy sulfates have been identified as the primary cause of fish mortality. Thus, the use of Biosurfactants

should be encouraged, since they are amphiphilic compounds that are produced on living surfaces, commonly seen on microbial cell surfaces. These compounds can also be released externally, where they aggregate as hydrophobic and hydrophilic components between different fluid phases.

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