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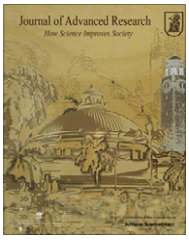

ORIGINAL ARTICLE

Experimental exposure of African catfish *Clarias Gariepinus* (Burchell, 1822) to phenol: Clinical evaluation, tissue alterations and residue assessment

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KEYWORDS

Phenol toxicity;
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Median lethal concentration;
Clinical Studies;
Pathology;
Tissue residue assay

Abstract There is lack of information regarding; the toxicological and pathological consequences of phenol stressed *Clarias gariepinus*; as well as; the susceptibility of the stressed fish to disease occurrence. Static renewal bioassay was experimentally conducted to evaluate the toxic effects of phenol on the African catfish *C. gariepinus*. Ninety-six-hour acute toxicity tests revealed that the median lethal concentration of phenol (LC50) is 35 mg/L by immersion. Four experimental fish groups were assigned for 3 weeks exposure test; three were exposed 20%, 50% and 70% LC_{50}, the fourth control fish group received a vehicle of dechlorinated water. Abnormal signs including cessation of feeding, nervous manifestations; skin expressed perfuses mucous, black patches with skin erosion and ulcerations in the later stages. All observations were correlated to the time and dose of exposure. Post mortem examination revealed adhesion of the internal organs. For tissue alterations; Skin, gills, brain, liver and kidney showed variable degrees of degenerative changes and necrosis. Muscle residues shown in mean ± SE were 4.3 ± 0.05 and 6.65 ± 0.05 ppm in groups that received 20 and 50% LD_{50}, respectively. Infection with *Aeromonas hydrophila* resulted in high percent of mortalities with a non significant difference between the challenged fish groups. The study cleared that phenol is toxic to *C. gariepinus* under experimental conditions.

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Introduction

The aquatic environment is a sink of toxic contaminants which find their way to the water bodies through industrial, domestic and agricultural activities [1]. These toxic chemicals disturb the integrity of the aquatic environment and adversely affect the aquatic animals [2].

Phenol and phenolic compounds are examples of toxic chemicals that act as endocrine disruptors; which mimic or antagonize hormones and disrupt the endocrine system. It also has great

potential for compromising the immune system and increases susceptibility of fish to secondary infections [3,4].

Phenols are discharged into water from the effluents of a variety of industries such as coal refineries, phenol manufacturing, pharmaceuticals, industries of resin, paint, dyeing, textile, leather, petrochemical, and pulp mill. Natural processes such as the decomposition of plant matter also contribute to phenol accumulations in the aquatic environment [5,6]. Phenols are of growing concern due to their high persistence and toxicity in the aquatic environment in addition to the difficulty in detecting them given their lack of taste and odor [7]. Unfortunately, there is a lack of information regarding phenol pollution and its effect in the Egyptian aquatic environment''.

Clarias gariepinus is one of the most abundant and widely distributed fish in the River Nile, its tributaries and lakes [8]. It is also the principal clarid catfish in Africa [9]. Pond-reared African catfish is at particular risk of exposure to agricultural chemicals, as they are often farmed in proximity to crop-producing fields using the resulting waste water [10]. The recorded level of phenol in Egyptian Waste Water was 0.05 PPM [11]. *C. gariepinus* was extensively used as a laboratory fish model by many scientists to monitor microbial, pathological or environmental studies [12]. Unfortunately, there is a lack of information about the toxicological and pathological consequences in *C. gariepinus* exposed to phenol.

The general aim of this study was to determine the impact of exposure of *C. gariepinus* to sublethal doses of phenol through; monitoring behavioral and clinical alterations, assessing the histopathological picture; as well as; investigation of phenol residues in fish muscles. Experimental infection of phenol challenged groups with *Aeromonas hydrophila* to monitor the concurrent exposure to pollutant and bacterial pathogens on the host level. Changes in these parameters are being discussed as potential diagnostic tools in assessing the effects of phenol on African catfish.

Material and methods

Experimental fish

A total of 150 sexually immature healthy African catfish *C. gariepinus*; [Average body weight 75 ± 5 g] were obtained from a semi-intensive aquaculture facility in the fish research station, World Fish Center, Abbassa, Egypt. The fish were free from any previous exposure to phenol either through therapeutics or water resources. Fish were transported in well aerated containers to the wet laboratory, the Department of Fish Disease and Management (FDML); Faculty of Veterinary Medicine; Cairo University Giza, Egypt. The fish were maintained for two weeks in glass aquaria with a stocking density of 7 fish/aquaria to be acclimated to the laboratory conditions at 23 ± 1 °C and under a 12 h light: 12 h darkness cycle, the measured physico-chemical water parameters were dissolved oxygen (DO) 5.65 ± 0.72 mg L^{-1}; pH: 8.2 ± 0.82; ammonia: 0.109 ± 0.024 mg L^{-1} at the onset of the experiments according to Clesceri et al. [13]. Fish were fed twice a day with a balanced commercial fish pelleted diet of 45% protein content (Zoo-control Company, Cairo, Egypt) [14]. Waste feed and fecal materials were suctioned daily.

Phenol

Pure grade of phenol (C_6H_6O) with a freezing point of 39.5–41.0 °C was obtained from Aldrich chemicals, Milwaukee, USA. Phenol aqua's solution was prepared by dissolving the estimated weights in water upon usage.

Determination of 96 h half lethal concentration (LC_{50}) of phenol

Seventy pre-acclimated catfish were held in 7 glass aquaria ($40 \times 80 \times 30$ cm^3) each containing ten catfish which represent a group. For the determination of 96 h LC_{50} of phenol, a static renewal acute toxicity bioassay test was conducted according to Behrines and Karber [15]. Each group of catfish was subjected separately to a renewed daily single dose of phenol; 0.0, 10 mg^{-1}, 20 mg^{-1}, 30 mg^{-1}, 40 mg^{-1}, 50 mg^{-1} and 60 mg^{-1}. The concentration which resulted in 50% mortality (LC_{50}) for 96 h exposure was calculated according to the following equation

$$LC_{50} = \text{Largest dose} - \sum \frac{A \times B}{N}$$

where A is a dose difference between two successive doses, B the mean of dead fish between two successive doses and N the total number of fish.

Experimental design for phenol exposure

Eighty catfish (for duplicate set of experimental work) were divided into eight glass aquaria ($40 \times 80 \times 30$ cm), each containing 40 L of static declorinated water and the aquarium holds ten catfish. Static experimental system was conducted at a constant water temperature (23 ± 1 °C).

The test fish were grouped into 8 equal groups, each phenol concentration was represented by 2 replicates. Each fish group was exposed by immersion to a single graded concentration of phenol (0.0%, 20%, 50% and 70% of the LC_{50}); which will be considered as test solutions; for 3 weeks as follows: Group 1 was kept in declorinated water containing 0.0% of phenol; served as the vehicle control; group 2, treated with 7 mg^{-1} in water (20% of the LC_{50}); group 3, treated with 17.5 mg^{-1} in water (50% of the LC_{50}) and group 4, treated 24.5 mg^{-1} in water (70% of the LC_{50}). As more than 30% of phenol is lost by volatilization [16,17], test solutions and water in the control were renewed daily. All the test exposures were carried out in duplicate.

Clinical and post mortem investigations

The phenol challenged fish were monitored closely during the experimental period. Behavioral responses, clinical signs and mortality rates were recorded daily according to Amlacher [18]. Post mortem examination was carried out on every dead fish during the experimental study and at the end of the experimental period.

Histopathological examination

By the end of phenol exposure period, tissues were dissected from challenged fish and processed for histopathological

examination. Briefly, samples from the liver, spleen, brain, kidney, skin (Skin was dissected from the skin flap under the dorsal fin) and gills were fixed in 10% neutral buffer formalin, processed by conventional methods and stained with hematoxylin and eosin according to Bancroft and Gamble [19]. The resolutions of the picture were corrected at 300 pixels. The slides were examined and captured using Olympus SZX12 microscope supplied by Olympus camera and monitor, USA.

Residual analysis of phenol

Representative pooled samples of 5 g dry weight were obtained from the dorsal muscles of 3 fish exposed to 20% and 3 fish exposed to 50% phenol LC_{50}. muscle samples were stored frozen in clean poly ethylene sealed capped tubes for determination of phenol residue. The samples were analyzed according to procedure recommended by AOAC [20]. Results were expressed in ppm dry weight of the tissue (mean ± SE). The residual analysis test was performed in Central Laboratory for Residue Analysis of Pesticides & Heavy Metals, Food Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt.

Experimental challenge of phenol exposed groups with A. hydrophila

To study the resistance of the tested catfish to *A. hydrophila* infection; a pathogenic local isolate of the bacterium from *C. gariepinus* ulcer disease was used in the bacterial challenge test. By the end of three weeks exposure to phenol; five *C. gariepinus* from each of the two intoxicated (20% and 50% phenol LC_{50}) groups, in addition, 5 control fish were injected with the bacterial culture and assigned as positive control; another 5 fish were kept without any treatment; in clean water; as the negative control;. Every fish group was reared in a separate glass aquaria. Fish from groups received 20%, 50% phenol LC_{50} and positive control were injected intraperitoneally with 0.1 mL PBS containing 10^8 live cells of a 24-h culture of *A. hydrophila*. The experiment was run in duplicate and all the groups were challenged on the same day. The percent of mortality was assessed up to 10 days after challenge [21]. The cause of mortality was confirmed by re-isolating the organism from the kidney of 10% of the dead fish.

Statistical analysis

The data were analyzed using a Chi-Square Test [22], where Probability ($P \leqslant 0.05$) was considered statistically significant.

Results

Determination of 96 h half lethal concentration (LC $_{50}$) of phenol

The calculated 96 h acute LC_{50} value of phenol, using a static bioassay system for African catfish was 35 mg/L. Results are depicted in Table 1.

Behavioral response clinical toxic signs and mortalities of phenol exposed catfish

The recorded mortality in the phenol exposure experiment was zero% in groups 1 and 2 (control and 20% of the LC_{50}), 10% in group 3 (50% of the LC_{50}) and 50% in group 4 (70% of the LC_{50}). The abnormal manifestations recorded were directly correlated to the time and dose of phenol exposure. The initial clinical signs; started 48 hr post phenol exposure; were difficulties in respiration manifested by increasing mouth movement and surfacing, in addition to nervous manifestations as vigorous erratic swimming abnormalities, surface to bottom movement, vigorous jerks and restlessness. The fish started showing gradual loss of appetite. Three days post exposure, black scattered spotted patches appeared on skin and fins with general skin paleness and wrinkling. Seven days post exposure; the fish displayed more prominent abnormal manifestations such as, skin erosion and ulcerations together with excessive mucous secretion. Fish stopped feeding and lacked signs of escape reflex as it was easy to catch nesting at the bottom and death. No behavioral changes or any mortality was recorded in the controls throughout the period of the bioassay. The behavioral, clinical and postmortem changes were noticeable among fish which received concentrations of (50% and 70%) and the least noticed at the group that received 20% concentration. Post mortem examination showed marked changes, mainly sunken eyes, tucked abdomen, pale gills, and adhesion of the internal organs, friable liver and pale kidney.

Histopathological finding

The histopathological changes in organs of the catfish exposed to different concentration of phenol showed lesions with variable intensity. The lesions were severe in fish treated with 70% of LC_{50} phenol whereas the fish treated with 50% LC_{50} phenol showed moderate lesions and the least changes were in the group that received 20% LC_{50} of phenol.

Table 1 The phenol concentrations, groups and calculations of the Acute 96 h LC_{50} value of phenol in African catfish *Clarias gariepinus*.

Phenol concentrations (mg/l)	No. of fish	No. of dead fish	A	B	aXb
0	10	0	0	0	0
10	10	1	10	0.5	5
20	10	2	10	1.5	15
30	10	4	10	3	30
40	10	6	10	5	50
50	10	7	10	6.5	65
60	10	10	10	8.5	85
	70				ΣaXb = 250

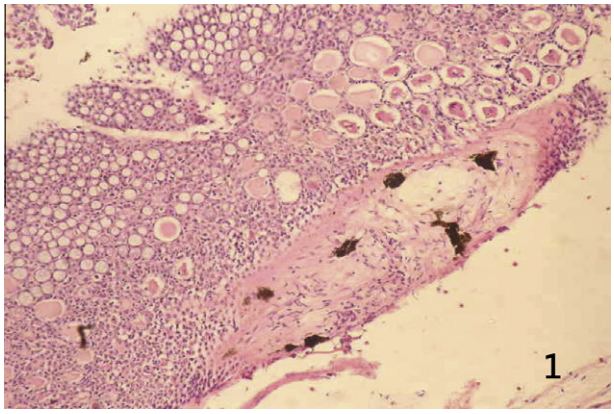

Fig. 1 Skin of African catfish exposed to 70% of LC_{50} of phenol showing hyperplastic activity of the epidermal layer especially the club cells and the mucous secreting cells (arrow) (stain H&E 100×).

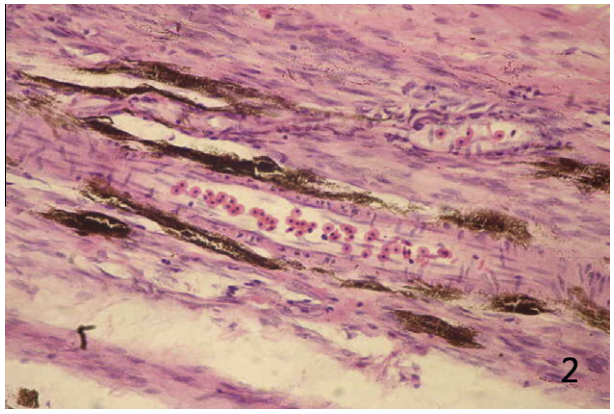

Fig. 2 Skin of African catfish exposed to 70% of LC_{50} of phenol showing increase in pigmented cells around the blood vessels (stain H&E 400×).

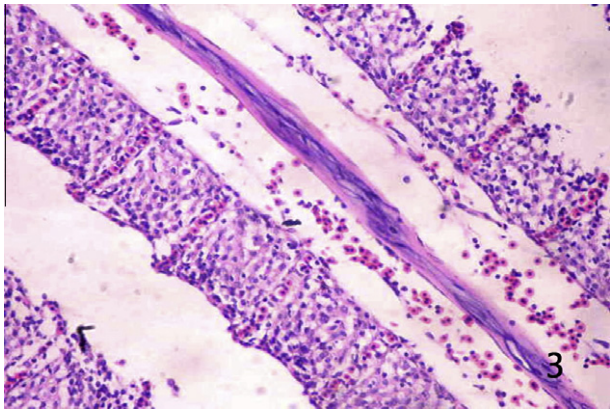

Fig. 3 Gills of catfish exposed to 70% of LC_{50} phenol, showing fusion of secondary lamellae with hyperplasia of epithelial lining (stain H&E 200×).

The skin showed hyperplastic activity of the epidermal layer especially the club cells and the mucous secreting cells (Fig. 1). There was an increase in pigmented cells in the dermis especially around the blood vessels and in the upper surface of

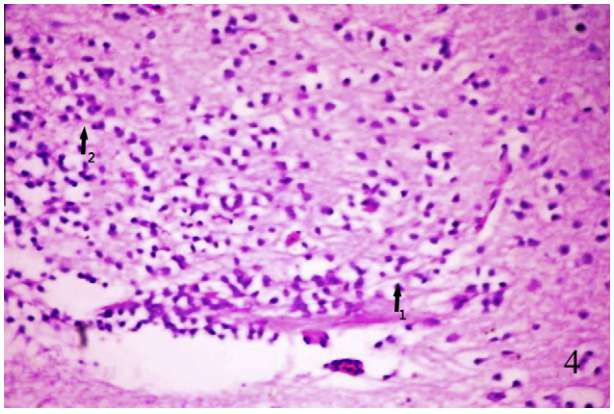

Fig. 4 Brain of African catfish exposed to 70% of LC_{50} of phenol showing cellular edema (arrow 1), degenerative changes and necrosis of nerve cells with neuronophagia (arrow 2) (stain H&E 200×).

dermis at its junction with the epidermis. Moreover some cases of fish exposed to 70% of LC_{50} phenol showed necrosis in the layers of epidermis (Fig. 2). The gills of fish exposed to 70% of LC_{50} showed fusion of the secondary lamellae (Fig. 3) as manifested by hyperplasia of its epithelial lining. There was also an increase in activity and number of the goblet cells. The brain showed cellular edema, degenerative changes and necrosis of nerve cells with neuronophagia and focal gliosis (Fig. 4).

The liver showed vacuolar degeneration in which the cells appeared swollen with vacuolation of their cytoplasm. (Fig. 5), some cases showed focal areas of necrosis especially in cases treated with 70% of LC_{50} phenol (result not shown). The renal tubular epithelium revealed various necrobiotic changes represented by hydropic degeneration and necrosis in fish exposed to 50% of LC_{50} (Fig. 6). The spleen showed a decrease in numbers of melano-macrophage centers with the absence of lymphocytes in fish exposed to 50% and 70% of LC_{50}.

Phenol residues

Phenol muscles residues shown in mean ± SE were 4.3 ± 0.05 and 6.65 ± 0.05 ppm in groups that received 20 and

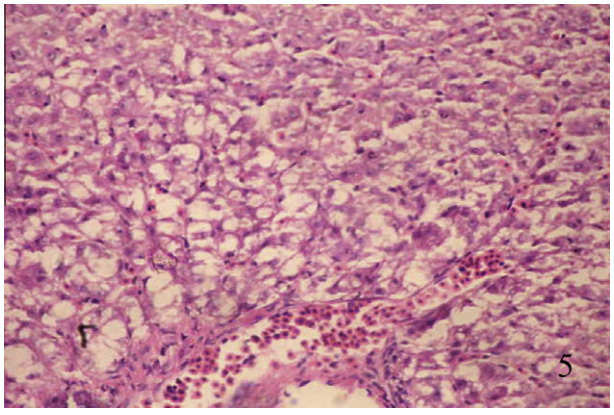

Fig. 5 Liver of African catfish exposed to 70% of LC_{50} of phenol showing vacuolar degeneration with congestion of sinusoids (stain H&E 400×).

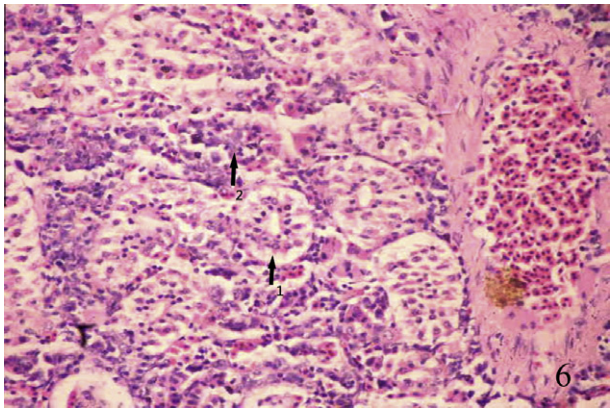

Fig. 6 Tubular epithelium of kidney of African catfish exposed to 50% of LC_{50} of phenol showing hydropic degeneration (arrow 1) and necrosis (arrow 2) (stain H&E 200×).

50%LD_{50}, respectively. The severity of the clinical picture as well as high mortality in the group 4 (70% of LC_{50}) did not encourage the estimation of phenol residues in such a group.

Results of experimental challenge infection of phenol exposure groups with A. hydrophila (Table 2)

Mortality started at the 3rd and peaked at the 5th day of post experimental infection with *A. hydrophila*. The number of dead fish and the recorded percent of mortalities for each group are recorded in Table 2. The cause of mortality was confirmed to be due to *A. hydrophila* infection by re-isolating the organism from the kidney of the challenged dead fish.

Chi squared values (χ^2) to test the significance difference in the percent of mortalities between different exposed groups were 4 and 1.7 respectively, which are non significant at probability 0.05.

Discussion

Rapid global industrialization and chemical pollutants have altered the natural condition of the aquatic medium, resulting in the functional imbalance of the aquatic organisms. Phenols are listed among the potent chemical toxicants adversely affecting the aquatic habitats.

The available literature for phenol toxicity did not give sufficient data regarding its LC_{50}, residual detection or pathology in the African catfish *C. gariepinus*, as well as its effect on clinical and behavioral parameters.

The estimated LC_{50} value of phenol to African catfish in the present study was 35 mg/L. In comparison; the recorded LC_{50} value of phenol in *Oreochromis niloticus* was 29 mg/L by Abdel-Hameid [23], 28 mg/L by Gad and Saad [24]. Acute toxicity of phenol was caused by 35.0 mg/l in *Oreochromis mossambicus* by Sannadurgappa et al. [25]. However LC_{50} of phenol to three Indian freshwater fish ranged from 12.53 to 39.40 mg/L by Verma et al. [26]. The current result indicated that African catfish seems to be more tolerant to phenol toxicity than many fish species.

Sublethal concentrations of toxicants in the aquatic environment will not necessarily result in outright mortality of aquatic organisms. However, the bioaccumulation of these pollutants over a period of time may constitute potential health hazards not only to aquatic organisms like fish (as applied in this study) but also on higher trophic level especially man. In the current study; the recorded results of mortalities in 3 weeks exposure were in accordance with Nair and Sherief [27] who reported mortalities in Juveniles of *L. rohita* 23 day post phenol exposure, mortalities occurred in a dose dependent manner. Saha et al. [28] studied the Toxicity of Phenol to *O. mosambecus* and recorded elevated mortalities with higher concentrations. Kobayashi et al. [29] investigated the toxicity of the chloro-phenols in goldfish and recorded that the mortality was enhanced with increasing concentrations.

From the onset of the exposure test and as it progress, catfish displayed elevated levels of physiological malfunctions. All of the signs were reciprocal to the concentration and duration of phenol exposure.

The perfuse skin mucous secretion was prominent in phenol intoxicated catfish. This can be explained by the fact that skin is among the first to be in close contact with the dissolved pollutants. Hence, reactions in the skin cells are spontaneous as a protection mechanism through increasing levels of mucous secretion over the body surface, forming a barrier between the body and the toxic medium, minimizing its irritating effect, thus, scavenge or even eliminates toxicants through the epidermal mucous. The results are consisted with those of Chebbi and David [30] and Ezemonye and Ogbomida [31].

All the skin tissue changes in the current study were subsequently reflected through the histopathological profile; the hyperplastic mucous cells in the epidermis of exposed fish could explain the clear increased level of mucous over the body surface as defense mechanisms of the body.

Respiratory manifestations recorded in phenol intoxicated groups are in accordance with Pandey et al. [32] who reported that introduction of toxicant into an aquatic system impaired the respiration through the decrease of the dissolved oxygen concentration leading to asphyxiation. Results obtained from

Table 2 The number of challenged fish, number of dead fish, and percent of mortality per treatment resulted from experimental challenge infection of *C. gariepinus* phenol exposure groups with *A. hydrophila*.

Group	Aquaria 1			Aquaria 2		
	No of fish	No of dead fish	Mortality (%)	No of fish	No of dead fish	Mortality (%)
Control negative	5	0	0	5	0	0
Control positive	5	2	40	5	2	40
20%	5	2	40	5	2	40
50%	5	4	80	5	3	70

Chi squared value (χ^2 value) for aquaria 1 is 4 which is non-significant at 0.05.
Chi squared value (χ^2 value) for aquaria 2 is 1.7 which is non-significant at 0.05.

the present work may be attributed to the direct effect of phenol that renders the medium unconducive for the fish, in addition to; the severe gill irritation resulted from phenol exposure. Moreover; gills; histologically; showed marked fusion of secondary lamellae due to hyperplasia of epithelial lining that aid in respiratory insufficiency. The well known excretory functions of gills add an additional negative impact of phenol on its tissue that aggravates respiratory impairment with great disturbance of gas exchange and ionic regulation. This opinion is consistent with that given by Chezhian et al. [33].

The abnormal nervous behaviors observed during the exposure in the current study were concomitant with those given by Chandra [34]. Such nervous manifestations could be attributed to; neuronal changes as well as the severe gill irritations caused by the chemical; consequently; phenol could be regarded as neurotoxic to exposed catfish.

Sublethal concentrations of pollutants in aquatic environments cause structural and functional changes in aquatic organisms. As the liver is the main organ for detoxification of organic xenobiotics. Hepatic damage of variable degree from phenol exposure is in agreement with those reported by Abdel-Hameid [16] who stated that liver changes were directly proportional with the concentration of phenol.

The last step for toxicant absorption; transportation and transformation is the excretion via kidney or gill. Kidney changes in the present study included alterations due to the direct damage during phenol excretion via the renal tubular epithelium. McKim et al. [35] confirmed the *in vivo* tubular secretion of phenolic compounds in freshwater adapted trout.

Muscles of catfish were targeted to estimate the residue in it as it can be the sources of phenol bioaccumulation in higher food chain organisms especially humans. The present study revealed that the concentrations of phenol in fish muscles were estimated as 4.3 ± 0.05 ppm and 6.65 ± 0.05 ppm in groups 2 and 3, respectively. Both were greater than the maximum permissible level which is 0.01 ppm in fish [36].

As many bacterial agents are considered to be opportunist pathogens, they cause disease in aquatic organisms only in association with stress [37], a greater understanding of the interactions between stress and disease occurrence is needed.

In the present study; the phenol intoxicated *C. gariepinus* groups evoked an elevated percent of mortalities when complicated with *A. hydrophila*. The results may be a completion to the generalized toxic picture of the stressed *C. gariepinus*; phenol exposure may alter the fish disease resistance and render it susceptible to disease. Alteration in the spleen; as an immune organ; was histopathologically evidenced; it expressed decrease in numbers of melano-macrophage centers with the absence of lymphocytes. Wester et al. [38] stated that, the density of melano-macrophage centers of the spleen may decrease in fish from contaminated waters leaving them immunocompromised and susceptible to the disease.

Although the mortality percentages in the experimentally challenged groups with *A. hydrophila* are numerically high, indicating its toxicity, non significant differences in the percent of mortalities between the challenged groups are statistically recorded. Future studies should be carried out to spot light on the possible impacts of lower concentrations of phenol on fish health in both acute and chronic manners.

The current results indicated that; exposure of fish to phenol could induce a negative impact on fish health and aggravates its susceptibility to infectious agents.

Conclusions

The present investigation studied the toxicity of phenol to African catfish, *C. gariepinus*, using three sublethal doses; 20%, 50% and 70% of LD_{50}. Mortality, behavioral and histopathological change, residual phenol analysis and the ability to resist experimental infection by *A. hydrophila* were some of the parameters monitored in the study. Changes in these parameters are potential diagnostic tools in assessing the effects of phenol on African catfish. Phenol was found to be toxic to African catfish *C. gariepinus* in time and dose dependent manner.

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