Removal of cadmium from freshwater cultured Nile tilapia *Oreochromis niloticus* using Neem Leave Water Extract (NLWE) and Neem Leave Powder (NLP)

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Abstract: The study aimed to investigate the clinical picture, bioassay of cadmium pollution evaluating the influence of Neem Leave Water Extract (NLWE) and Neem Leave Powder (NLP) on recovery and removal of cadmium in tissues of Nile tilapia *Oreochromis niloticus* and water. *Azadirachta indica* (A. indica) leaves were obtained and Leave Water Extract (NLWE) and Neem Leave Powder (NLP) were prepared. To evaluate effect of neem leaf as water extract (NLWE) and neem leaf powder (NLP). A total of 80 apparently healthy Nile tilapia *Oreochromis niloticus,* weight (80±5) gram, fish were divided into four groups of 20 fish each, comprising three experimental groups and one control. 1st group was control simultaneously exposed to dechlorinated tap water only. 2nd group was exposed for cadmium chloride (15 mg/l). 3rd group exposed neem leaf water extract (1/10 LC50) only. 4th group exposed to cadmium chloride (15mg/l) and neem leaf powder NLP (200g/l). Clinical signs, post mortem lesions and mortality were monitored and recorded. Blood was collected from five of fish in each group after 30 days of starting the experiment. The musculature, skin, gills, liver, spleen and kidneys were collected from five fish of each group after the period of experiment for histopathological examination. From the present study, it was concluded that, Neem leave powder NLP efficiently remove cadmium from water decreasing it in tissues of fish. while NLWE remove cadmium in low degree but affect significantly the hematological, physiological and immunological state of *O. niloticus*, improving health status of fish.

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Key words: cadmium; (NLWE); (NLP); *Azadirachta indica; Oreochromis niloticus;* Clinical signs; physiological; immunological.

Introduction:

There is a great concern about the toxic impacts and environmental pollution caused by heavy metals especially in aquaculture all over the world. Toxic heavy metal ions get introduced to the aquatic streams by means of various industrial activities viz. mining, refining ores, fertilizer industries, tanneries, batteries, paper industries, pesticides etc. and posses a serious threat to environment. (**Dhiraj Sud *et al*.,2008**). Cadmium accumulates in the different organs causing fish death in many cases due to the more susceptibility to the bacterial infections and impairment of the immune system (**Baldisserotto *et al*., 2006)**.

Conventional techniques have their own inherent limitations such as less efficiency, sensitive operating conditions, production of secondary sludge and further the disposal is a costly affair (**Ahluwalia and Goyal, 2005a**). Another powerful technology is adsorption of heavy metals by activated carbon for treating domestic and industrial waste water (**Hosea *et al*., 1986). The** utilization of sea weeds, moulds, yeasts, and other dead microbial biomass and agricultural waste materials for removal of heavy metals has been explored (**Bailey *et al*., 1999; Sudha and Abraham, 2003).**

Recently attention has been diverted towards the biomaterials which are by products or the wastes from large scale industrial operations and agricultural waste materials.Neem, Azadirachta indica (*A. indica*) is one of the most promising medicinal plant, having a wide spectrum of biological activity, well known mainly for its insecticidal properties (**ICAR, 1993**). Every part of neem tree have been known to possess a wide range of pharmacological properties, especially as antibacterial, antifungal, antiulcer, repellent, pesticidal and detoxifying agent (**Biswas *et al*., 2002; Das *et al*., 2002; Mousa *et al*., 2008**). One of the most promising natural compounds is azadirachtin (AZA), an active compound extracted from the neem tree (*Azadirachta indica*), whose antiviral, antibacterial and antifungal properties have been known for 2000 years **(Isman *et al*., 1990; Harikrishnan *et al*., 2003)** Neem has been used successfully in aquaculture systems to control fish predators and treatment of large numbers of bacterial and parasitic fish diseases (**Dunkel and Ricilards, 1998; Mona *et al*., 2011).**

Present study aimed to investigate the clinical picture, bioassay of cadmium pollution evaluating the influence of Neem Leave Water Extract (NLWE) and Neem Leave Powder (NLP) on recovery and removal of cadmium in tissues of Nile tilapia *Oreochromis niloticus* and water with monitoring some blood, physiological parameters and histopathological alterations due to exposure to cadmium and Neem water extract (NLWE) and Neem Leave Powder (NLP).

Material and methods:

Experimental fish:

A total of 100 apparently healthy Nile tilapia *Oreochromis niloticus*, weight (80±5) gram obtained from private fish farm and acclimated for 2 weeks in aquaria supplied with dechlorinated tap water with continuous aeration.

Preparation of Neem Leaf Water Extract (NLWE) and Neem Leave powder (NLP):-

Azadirachta indica (*A. indica*) leaves were obtained from nurseries of agricultural ministry, Giza, Egypt dried and finely chopped, grounded in blender then amount of 500g was soaked in tap water, (liter of water) for 24 h at room temperature as described by **Cruz *et al.,* (2004)**. The mixture was filtered and the extract (500 g/l) was used immediately in the experiments as. (NLWE). While the reset of grounded leave used as it is as (NLP).

Clinical and post mortem examination:

Clinical and post mortem examination was carried out to the fish (*O.niloticus*) exposed to cadmium and neem each alone and cadmium and neem (neem water extract and neem leave powder) together according to **(lucky, 1977**).

Determination of 96-h LC50

Static toxicity tests were run to determine lethal concentrations (96-h LC50) of neem leaf water extract to *Oreochromis niloticus* fish, tests were conducted in 30 L glass aquaria, 6 fish per aquarium, containing neem leaf extract diluted in tap water to the following concentrations: 0 (control group), 1, 2,4,6,8,10, 12, g /l. Each treatment had 3 replicates. All laboratory conditions were maintained constant. Deaths and abnormal behavior fish were recorded every 3 h for the 1st day, then every day for other 3 days. The value of 96-h LC50 were estimated.

Experimental design:

To evaluate effect of neem leaf as water extract and neem leaf powder. A total of 80 apparently healthy Nile tilapia *Oreochromis niloticus*, weight (80±5) gram fish were divided into four groups of 20 fish each, comprising three experimental groups and one control. Each group was placed in separate glass aquaria. 1st group was control simultaneously exposed to dechlorinated tap water only. 2nd group was exposed for cadmium chloride (15 mg/l) (**Osman *et al.*, 2009**). 3rd group exposed neem leaf water extract (1/10 LC50) 0.2 ppm only. 4th group exposed to cadmium chloride (15mg/l) and neem leaf powder NLP (200g/l). The experiment was carried out in static systems. Clinical signs, post mortem lesions and mortality were monitored and recorded during treatment.

Table 1: Cadmium exposure and treatment with (NLWE) and (NLP)*** (experimental design)**

Groups*	Cadmium exposure	Concentration of Neem
1st (negative control)	0	0
2nd (positive control)	15	0
3rd Treated group NLWE	15	0.2 ppm
4th Treated group NLP	15	55 g/l

* Each group 20 fish

**NLWE= Neem water extract

***NLP= Neem leave powder

Blood sampling:

Blood was collected from the caudal vein of five fish in each group after 30 days of starting the experiment. The liver, kidneys, musculature and gills were also collected from five fish of each group after the period. The blood sample was divided into two portions. The first portion was kept as a whole blood in heparinized tubes for hematological examination. Serum was separated from the second portion for biochemical analysis. Tissue homogenates were prepared from liver, kidneys, musculature and gills were digested as described by **Cottenie (1980)** for determination of cadmium residues.

Tissue Cadmium Determination:

Cadmium concentration was determined in the tissues according to **Jackson (1973)**.

Hematological Examination:

Packed cell volume (PCV), hemoglobin (Hb) concentration and red blood cell (RBC) count were examined in the whole blood as described by **Stoskopf (1992).**

Biochemical Analysis

Serum:

Total protein level in serum was determined according to **Cannon *et al.* (1974)** Serum albumin concentration was measured as described by **Gustafsson (1976)**. Blood serum globulin was calculated by subtracting the concentration of albumin from that of the total protein and albumin/globulin ratio (A/G ratio) was calculated by dividing albumin concentration over that of globulin **Coles (1986).**

Histopathological examination:

The macroscopic lesions of internal organs (Liver, kidneys, musculature and skin of all groups were collected and fixed in 10% formal saline for histopathological examination). Small pieces of suspected lesions of the organs were taken and preserved in 10% formalin for 24 hrs. Paraffin section (5-10 microns in thickness). Liver and musculature of infested fish after fixation in 10% formalin solution. Sections were stained with haematoxylin and eosin stain (H&E). **Bancroft *et al*. (1996**).

Statistical Analysis:

Data were presented as mean±standard error (S.E.) and the significance of differences was estimated using ANOVA test (**Senedecor, 1964**).

Results:

96-h LC50 for *O.niloticus:*

96-h LC_{50} for *O. niloticus* exposed to NLWE was 2 ppm while NLP was 550 gm (table 1).

Table 1. Lethal and sublethal concentrations of Neem leaf aqueous extract NLWE (ppm) and Neem leaf powder NLP (gm) for Nile tilapia,

Neem form	96-h LC_{50}(g/l)	$^{1}/_{10}$ LC_{50}(g/l)
Neem leaf as water extract NLWE	2 ppm	0.2 ppm
Neem leaf powder NLP	550 gm	55 gm

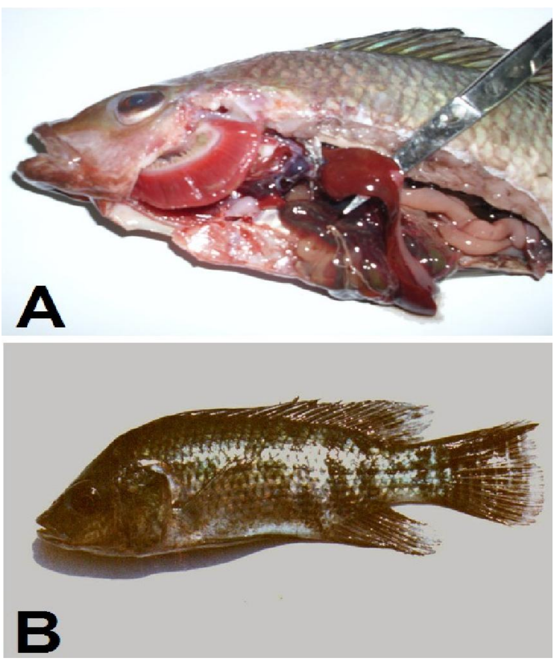

Figure 2: Showing (A) *O. niloticus* exposed to cadmium showed paleness and excessive mucous on gills with inflammation and enlargement of spleen, and liver (B) *O. niloticus* showing dark metallic skin with excessive slimness exposed to cadmium chloride

Clinical signs of fish:

O.niloticus exposed to NLWE showed respiratory distress, gasping, gulping the atmospheric air erratic swimming with some nervous manifestations in the form of fish moved in all directions of aquaria and some of them swam in circular directions were noticed during determination the LC50 of NLWE, while fish exposed to NLP being normal without any abnormal sings, cadmium showed slimy body with dark skin colour (fig1,B), with signs of restlessness some fish suffered from asphyxia and jumped outside water, finally loss of appetite, escape reflex and settle down to the bottom, sluggish movement.

Post mortem of fish:

O.niloticus exposed to NLWE showed congested gills, distended, enlarged gall bladder, while fish exposed to NLP showing no lesions while fish exposed to cadmium showed paleness and excessive mucous on gills with inflammation and enlargement of spleen, enlargement and distended gall bladder with spotted inflammatory patches in the liver (fig1,A).

Histopathological Examination:

The histopathological alterations were observed in musculature as suffered from hyalinization of some muscular bundles with infilteration of lecuocytic inflammatory cells with diffused deposition of melanin pigmented cells (fig 3 A &B). gills suffered from advanced stages of hyperplasia, odema of the core of primary gill lamellea (fig 3C&D). Spleen was manifested as hemosiderin was detected in the congested red pulps focal melanin pigment cells deposition was observed in the white pulps and in the perivascular tissue of the dilated and congested blood vessels (fig4, E).

The kidney was manifested as focal haemorrhage in between the degenerated and necrosed tubules associated with dilatation and congestion in the blood vessels with perivascular deposition of melanin pigmented cells (fig 4, F) The liver was manifested as melanin pigmented cells with leucocytes inflammatory cells infiltration were observed in the portal vein associated with congestion in the central vein (fig 4,G &H).

Table 2: Cadmium residues (mg/g wet weight) in the organs of *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with NLWE and NLP

Organ / group	liver	kidney	muscle	gills
Control	0.041±0.002 A	0.043±0.002 A	0.031±0.001 A	0.061±0.002 A
Cadmium	6.280±0.170 aB	3.215±0.128 aB	1.286±0.067 aB	1.276±0.073 aB
Cadmium+ 0.2 mg/l NLWE	5.940±0.164 aC	2.995±0.106 aC	1.178±0.072 aC	1.210±0.039 aC
Cadmium+ (55 gm) NLP	2.990±0.105 abc	1.592±0.067 abcd	0.432±0.013 abcd	0.764±0.023 abcd

Each value represents mean ± S.E.; N=5.
Small letters a, b, c and d in the same column represent a significant change against capital letters A, B, C and D respectively by LSD using ANOVA at P= 0.05

Table 3: Some hematological prarameters in *Oreochromis niloticus* exposed to 15 mg/L cadmium and treated with NLWE and NLP

Parameter / group	RBC count (x10^6/mm)	Hemoglobin	Pcv %
Control	3.20±0.10 A	8.32±0.30 A	25.60±1.41 A
Cadmium	1.70±0.07 aB	5.52±0.17 aB	17.62±1.29 aB
Cadmium+ 0.2 mg/l NLWE	1.80±0.08 aC	5.83±0.19 aC	19.65±1.26 aC
Cadmium+(55 gm) NLP	2.15±0.08 bc	7.18±0.24 bc	24.40±1.19 bc

Each value represents mean ± S.E.; N=5.
Small letters a, b, c and d in the same column represent a significant change against capital letters A, B, C and D respectively by LSD using ANOVA at P= 0.05

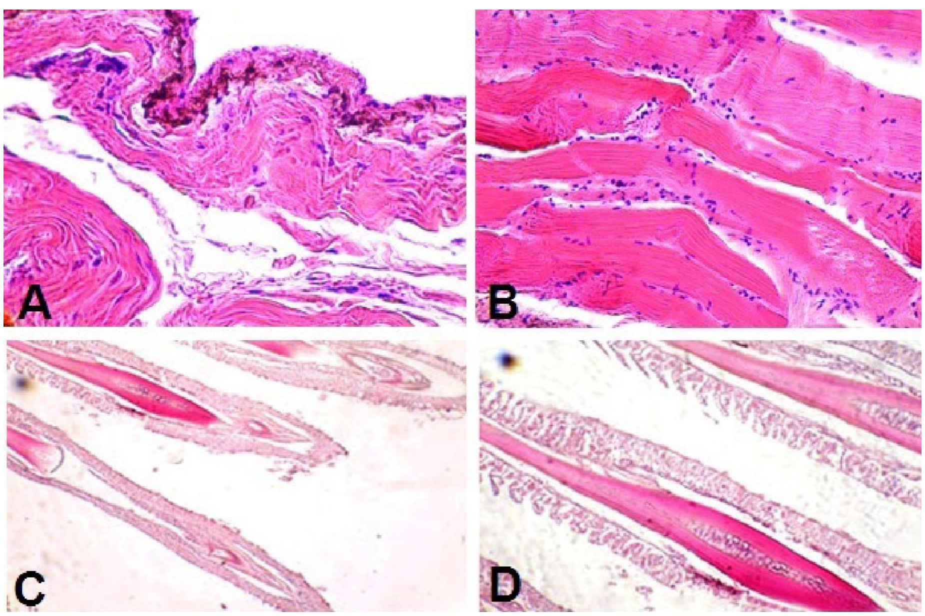

Figure 3; Showing (A &,B) skin and musculature of *O.niloticus* exposed to cadmium: there were diffused melanosis with inflammatory cells infiltration in the dermis, in association with hyalinization of some skeletal muscle bundles (C& D), hyperplasia of secondary gill filaments with edema of the core of primary gill lamellae

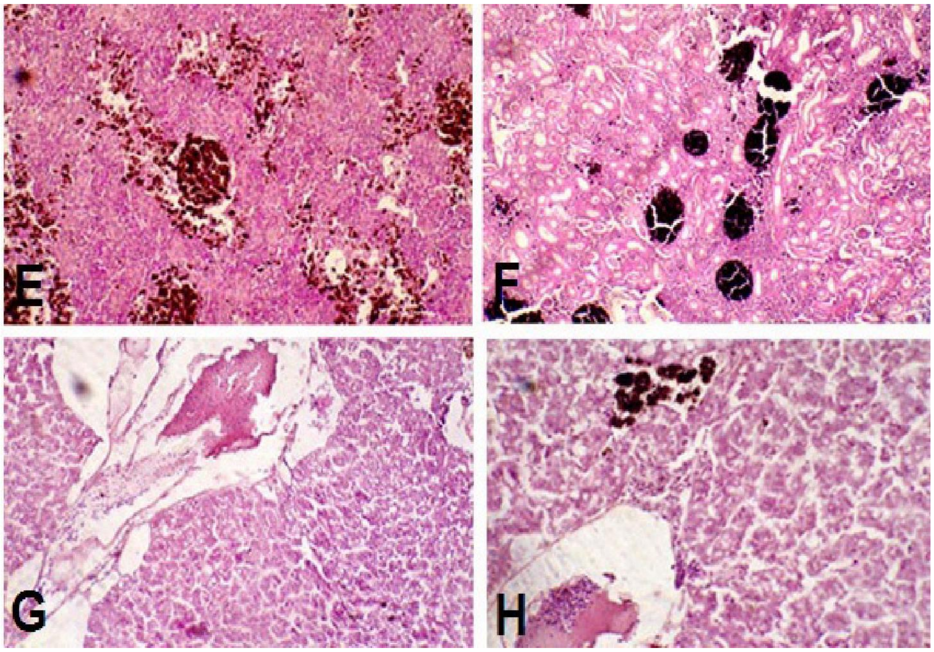

Fig 4: Showing (E& F) spleen and kidney suffered from focal melanosis and necrosis of cells of spleen and kidney, (G&H), liver of *O.niloticus* exposed to cadmium showing congestion in the central vein and sinusoids with hemosiderosis, with necrosis and degenertation in the hepatocytes,

Table 4: Blood serum proteins levels in *Oreochromis niloticus* exposed to 15 mg/l cadmium for 30 days and treated with NLWE and NLP

Parameter / group	Total protein g/dl	Albumin g/dl	Globulin g/dl	A/G ratio
Control	4.75±0.28 A	1.63±0.08 A	3.12±0.15 A	0.59±0.03 A
Cadmium	3.15±0.14	0.88±0.04 aB	2.27±0.10 aB	0.32±0.02 aB
Cadmium+0.2mg/l NLWE	3.30±0.15	0.96±0.05 Ac	2.34±0.10aC	0.37±0.02 aC
Cadmium +(55 gm) NLP	4.64±0.25	1.56±0.08 bc	3.08±0.13 bc	0.55±0.04 bc

Each value represents mean ± S.E.; N=5.
Small letters a, b, c and d in the same column represent a significant change against capital letters A, B, C and D respectively by LSD using ANOVA at P= 0.05

Discussion:

present study investigate efficacy of Neem leave water extract (NLWE) as detoxifying agent and Neem leave powder (NLP) as adsorbent agent in removal of cadmium in water and *O. niloticus* tissues and as immunostimulants in improving the physiological and immunological status of *O.niloticus*. Neem leaves being economic and ecofriendly due to their natural origin, availability in abundance, renewable, low in cost and more efficient are seem to be viable option for heavy metal remediation. Studies reveal that various agricultural materials such as rice bran, rice husk, wheat bran, wheat husk, saw dust of various plants, bark of the trees, groundnut shells, coconut shells, black gram husk, hazelnut shells, walnut shells, cotton seed hulls, waste tea leaves, etc has been tried (**Annadurai *et al*., 2002; Mohanty *et al*., 2005; Dhiraj Sud *et al*.,2008**) development of modern drugs from neem should be emphasized for the control of various diseases. An extensive research and development work should be undertaken on neem and its products for their better economic and therapeutic utilization (**Schmutterer, 1995 & Ketkar and Ketkar, 1995**). **Bhattacharyya and Sharma (2005)** and **Serafini Immich *et al*., (2008)** investigates the efficiency of neem tree leaves powder in the removal of Remazol Blue RR present in aqueous solution, they studied color removal from textile effluent through adsorption processes using Neem leaf powder (Azadirachta indica) as an adsorbent. They reported that there was high efficiency of Neem leaf powder (NLP) in the color removal process. **Serafini Immich**

et al**., (2008)** added that The results given showed that the natural adsorbent Neem, without the extract, is not toxic toward the aquatic environment Therefore, this adsorbent can be used in the adsorption processes without adverse or toxic effects on aquatic organisms.

Concerning to the clinical sings and post mortem lesions appeared on fish after exposure to cadmium and neem leave water extract each alone *O.niloticus* exposed to NLWE showed respiratory distress, gasping, gulping the atmospheric air erratic swimming with nervous manifestations in the form of fish moved in all directions of aquaria and some of them swam in circular directions were noticed while fish exposed to cadmium showed slimy body with pale skin with signs of restlessness some fish suffered from asphyxia and jumped outside water, finally loss of appetite, escape reflex and settle down to the bottom, these results nearly agree with the results obtained by **Mousa *et al*., (2008); Osman *et al*.(2009) and Mona *et al*.(2011).**

Regarding to the histopathological alterations due to exposure to cadmium pollution, the study revealed that the affected liver was manifested as melanin pigmented cells with leucocytes inflammatory cells infiltration were observed in the portal vein associated with congestion in the central vein. Beside that kidney was manifested as focal hemorrhage in between the degenerated and necrosed tubules associated with dilatation and congestion in the blood vessels with perivascular deposition of melanin pigmented cells. In addition, the affected gills was manifested with destruction in most of the lamellae in other filaments and hyperplasia in some other filaments and lamellae. There was diffuse haemorrhage allover the arch, while the racker showed goblet cells formation and oedema in the connective tissue core, musculature was manifested as hyalinization in some muscular bundles and spleen was manifested as hemosiderin detected in the congested red pulps focal melanin pigment cells deposition was observed in the white pulps and in the perivascular tissue of the dilated and congested blood vessels these results nearly agree with **Oliveira Ribeiro *et al*. (2002), Thophon *et al*. (2003); Gupta and Srivastava (2006); Kaoud and El-Dahshan (2010).**

Concerning the concentration of cadmium in fish tissues it is observed that cadmium concentration in liver, kidney, musculature and gills was significantly higher in fish exposed to cadmium for 30 days than control group and the elevation in cadmium concentration is more drastic at the end of 30 days exposure. The treatment with 55 g/l NLP or 0. 2 g/l of NLWE concentrations significantly decreased the concentration of cadmium in tissues but not to the level of control. Present results indicate that 200 g/l NLP is effective in decreasing the adverse effect of Cd pollution removing it from water reducing Cd bioaccumulation in fish,its effect was pronounced than NLWE. Particulate organic matter can scavenge metal from water and help to reduce metal from fish. These results are in agreement with **Santachi (1988)** who found that any agent that can remove Cd from water helps to reduce the bioaccumulation of this metal in fish. Cd accumulation in liver, gills and musculature of fish exposed to Cd alone was higher than that of NLP treated group and NLWE treated group. These results suggest that NLP and NLWE could remove Cd ions producing a stable complex, thus reducing the chance for metal uptake by tissues. Besides, that neem in its two forms can eliminate more amount of Cd from the body through secretions of feces and urine.

Regarding hematological parameters, cadmium exposure for 30 days significantly diminished RBC count, PCV and hemoglobin concentration in *Oreochromis niloticus* in comparison with control. Treatment with NLWE at concentrations 0.4 or 0.2 g/l significantly elevates these parameters near the control values. The reduction of these parameters in Nile tilapia*, O. niloticus* exposed to cadmium might be due to the destruction of mature RBCs and the inhibition of erythrocyte production due to reduction of haemosynthesis that affected by pollutants **Wintrob (1978).** Also, the decrease in RBCs count may be attributed to haematopathology or acute haemolytic crisis that results in severe anemia in most vertebrates including fish species exposed to different environmental pollutants **Khangarot and Tripathi (1991)** or may be the decrease in the RBCs may be attributed to reduction of growth and other food utilization parameters which results in sever aneamia **(James and Sampath 1999**; **Kaoud *et al*.,2011**). Also, **Gill** and **Epple (1993**) found a significant reduction in the RBCs, Hb and Hct in American eel Anguilla rostrata after exposure to 150 µg Cd/L. **Karuppasamy *et al*. (2005**) found a significant decrease in total erythrocyte count, haemoglobin content, haematocrit value and mean corpuscular haemoglobin concentration in air breathing fish, *Channa punctatus* after exposure to sublethal dose of Cd (29 mg CdIL).The addition of NLWE increased in concentration the haematological parameters (RBCs, Hb and Hct) which indicating to the capability of NLWE to chelate Cd from the water and fish subsequently, the Cd pollution was reduced. These results are in agreement with **Osman *et al*.(2009)** who observed that *Oreochromis niloticus* exposed to cadmium along with Humic acid as chelating agent showed a significant improvement in blood parameters due to reduction of cadmium level in water and fish.

Respecting the serum protein, cadmium exposure for 30 days significantly declined the levels of total protein, albumin and globulin as well as A/G ratio comparing with control. Decreases in serum protein concentration and the albumin/globulin ratio in the blood may indicate some liver dysfunction. When exposed to stressors, the gills become "leaky" to water and ions, often resulting in osmoregulatory imbalance **Mazeaud *et al*. (1977).** So the decline in serum total protein, albumin and globulin may be also due to a degree of haemodilution under the stress of pollution. The A/G ratio is an index used to track relative changes in the composition of serum or plasma **Jacobes *et al*. (1990).** A drop in A/G ratio can indicate a shift from albumin production to globulin proteins in response to stress. The treatment with 0.4 or 0.2 g/l of NLWE in the aquarium water significantly elevates the values of these parameters to nearly the values of control. These may be due to that presence of NLP as a chelating agent reducing cadmium level from water as well as fish tissues improving physiological status and enhancing immune response of fish. **(Khan and Wassilew, 1987)** who reported that the aqueous extract of neem bark and leaf also possesses anticomplement and immunostimulants activity and Neem oil has been shown to possess activity by selectively activating the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge. The results of the present study nearly agree with **Chandra and Khuda-Bukhsh (2004)** who mentioned that the $Cdcl_2$, is a common pollutant, and Aza, a natural product of the neem plant used extensively as an 'ecofriendly' agent for many purposes, had any genotoxic effect of their own on nontarget aquatic organisms of economic importance; and second, if Aza could have any ameliorating effect on $Cdcl_2$-induced genotoxicity in *O. mossambicus* tissues. As compared with distilled water-treated controls, both $Cdcl_2$ and Aza induced genotoxicity in *O. mossambicus,* the former in greater quantity than that produced by Aza. However, Cd-induced toxicity in *O. mossambicus* appeared to be ameliorated to some extent by Aza.

From the present study, it was concluded that, Neem leave powder NLP efficiently remove cadmium from water (as adsorbance) decreasing it in tissues of fish improving aquaculture an ecosystem of fish, it is not toxic toward the aquatic environment Therefore, this adsorbent can be used in the adsorption processes without adverse or toxic effects on aquatic organisms while NLWE remove cadmium in low degree but affect significantly the hematological, physiological and immunological state of *O. niloticus*, improving health status of fish. So Neem as natural product is a promising tool for controlling cadmium pollution in aquaculture when it used in two forms NLP or NLWE., Also, they significantly reduces cadmium level in fish tissues including musculature.

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