

Cytoprotective effect of *Nelumbo nucifera* and *Aegle marmelos* in Common Carp (*Cyprinus carpio* L.) exposed to heavy metals

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Abstract

The aim of the current study is to investigate the antioxidant properties and cytoprotective effect of *Nelumbo nucifera* (500mg/Kg diet) and *Aegle marmelos* (500mg/Kg diet) in comparison to standard reference compound silymarin (100mg/Kg diet) on combined heavy metals (Cd+Pb+Ni+Cr) induced oxidative stress in liver, kidney and blood of common carp (*Cyprinus carpio* L.). Thiobarbituric acid reactive substances (TBARS) are found to significantly increase in liver and kidney. Conversely all antioxidant enzymes, vitamin C and vitamin E are found to decrease on 32nd day in heavy metal exposed fish compared with the control groups. Treatment with *Nelumbo nucifera* and *Aegle marmelos* marks a highly significant increase in antioxidant enzymes. The results confirm the cytoprotective and antioxidant potency of *Nelumbo nucifera* and *Aegle marmelos* by the stabilization of plasma membrane and modulation of antioxidant enzyme systems against stress induced by heavy metals in *Cyprinus carpio* L.

Keywords: Antioxidant enzymes, *nelumbo nucifera*, *aegle marmelos*, heavy metals, liver, kidney, *Cyprinus carpio* L..

INTRODUCTION

Environmental conditions and human influence has greatly accelerated the rate of environmentally deleterious changes by continuously loading water systems with chemical xenobiotics. Fish are considered an important food source for human beings because their flesh contains a high percentage of protein, calcium and phosphorus. Fish serves as a bioindicator species in monitoring the water pollution (Whitfield and Elliot, 2002). The sudden death of fish indicates heavy pollution with multiple contaminants that are dissolved in water and cause bioaccumulation in the tissues and organs of fish. Various substances are known to cause liver and kidney damage and the most noxious factor are heavy metals which are well known hepatotoxins and nephrotoxins (Adami *et al.*, 2002; Filipovic and Raspor, 2003; Coen, 2001; Banerjee and Devi, 2007). The fact that heavy metals cannot be destroyed by biological degradation and have the ability to accumulate in the environment make these

toxicants deleterious to the fish and consequently to humans who depend on aquatic products as a source of food (Ashraf, 2005).

The effect of exposure to sub-lethal levels of heavy metals can be measure in terms of biochemical, physiological or histological responses of the fish (Mondol *et al.*, 2001; Siroka and Drastichova, 2004). Biochemical markers have been used by the employment of in vivo tests in aquatic environment to assess the health status of fish (Schlenk and Digiulio, 2002). Heavy metals have been reported to accumulate in the tissues of fish and exert oxidative stress which can lead to redox reactions generating reactive oxygen species. This damaged DNA, proteins, lipids and other molecules. The impact of free radicals can be counterbalanced by generating antioxidant enzyme systems such as superoxide dismutase, catalase and glutathione family proteins as detoxifying agents towards lipid hydroperoxides (Farombi *et al.*, 2007).

However, most of existing evidence indicates that the endogenous production of antioxidants is insufficient to balance the oxyradicals. The effective method for minimizing the formation of reactive oxygen species and strengthening natural defensive systems by administration of natural substances that acts as antioxidants or free radical extinguishers (Kubinova

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and Suchy, 1999; Bartosikova *et al.*, 2003). Plants are natural resources yielding valuable herbal products which are often used in the treatment of many ailments such as liver disease, cardiovascular problems and metabolic disorders. Moreover, 11% of 252 drugs considered to be basic and essential by the WHO are isolated from plant sources and used directly (Rates, 2001). The use of crude plants, plant extracts and purified compounds provide the foundation to modern medicine in the discovery of new drugs, chemotherapeutic agents and health care products (Cos *et al.*, 2006).

The prophylactic and therapeutic efficacy of herbal formulations for the management of degenerative changes has been found to be a reservoir of pharmaceuticals (Arora *et al.*, 2003; Krishnaraju *et al.*, 2006). Two such medicinal plants *Nelumbo nucifera* and *Aegle marmelos* invite attention for their biopotency and their tissue regeneration capacity in management of different disorders.

Nelumbo nucifera geartn (Nymphaeaceae; Kamal; lotus) is an aquatic herb commonly found in large lakes and temple ponds in all over India. The plumule portion of *Nelumbo nucifera* has antioxidant (Wang *et al.*, 2003) and anti-inflammatory effect (Huang and Wu, 2002). The embryo of flower exhibits a cardioprotective effect (Malakul *et al.*, 2001), hypoglycemic (Mukherjee *et al.*, 1997) and hypolipidemic effects (Lacour *et al.*, 1995).

Aegle marmelos commonly known as bael, is a spiny tree belonging to the family Rutaceae. All parts of tree are edible and have medicinal values. *Aegle marmelos* leaf possess contraceptive (Bhattacharyay, 1982); cardiogenic (Nadkarni, 2000; Rajadurai and Prince, 2005); Hypoglycemic (Sabu and Ramadasan, 2004); antiperoxidative (Rajadurai *et al.*, 2005) and antispermatogenic activity (Sur *et al.*, 2002).

The rich heritage and diverse medicinal properties of these two herbals *Nelumbo nucifera* and *Aegle marmelos* attracted attention in the search for better protection and immunological tolerance in mitigating metal toxicity and in decreasing the incidence of oxidative damage in heavy metal exposed aquatic environments.

Common carp (*Cyprinus carpio* L.) is an important commercial species around the world to feed populations and is an economic rather than an ornamental fish. Reducing the availability of these fish to meet heavy demand as food due to metal toxicity is unconscionable. Our previous study reported the bioaccumulation of heavy metals in gills, liver, kidney and flesh (Vinodhini and Narayanan, 2008b). The present study attempts to evaluate the antioxidant and cytoprotective nature of *Nelumbo nucifera* and *Aegle marmelos* compared with standard reference compound

silymarin (*Silybum marianum*; Compositae) in combined heavy metals exposed aquatic system using common carp as an experimental model.

MATERIALS AND METHODS

Preparation of herbal drugs

Fresh leaves of *Aegle marmelos* and the flower petals of *Nelumbo nucifera* were collected from southern district of Tamil Nadu, India. The leaves and flower petals are washed in distilled water to remove the dirt and air dried at 45°C for 48hrs, powdered using an electrical grinder and passed through test sieves of 200mm in diameter and 250µm in aperture to collect the fine powder and stored airtight in plastic polyethylene bottles. This fine crude powder of *Nelumbo nucifera* and *Aegle marmelos* served as herbal drugs for the study.

Selection of experimental animals

Freshwater common carps (10–13cm long and weighing 35.70±0.60g) were collected from southern districts of Tamilnadu, India and were acclimatised to laboratory conditions for a week. The department animal ethical committee has given the clearance to carryout the experiments. All fish were kept in plastic aquariums of 200lit capacity containing well aerated unchlorinated tap water before they were used for the experiments. Forty to forty-five fish in total were used for the experiment. The feeding, maintenance of the fish and the physiochemical characteristics of water used for acclimatisation, control, experimental pond and concentration of heavy metal intoxication are provided elsewhere. (Vinodhini and Narayanan, 2008a).

Experimental design

The fish were divided into eight groups. Control fish (group I) received normal feed and survive in tap water throughout the experiment. Experimental fish (group II–V) were exposed to sublethal concentration of 5ppm of combined (Cd+Pb+Cr+Ni) metal solution containing 1.25ppm of each metal ion (1/10th of LC 50/ 48h) for a period of 1, 8, 16 and 32 days (Vinodhini and Narayanan, 2008a). At the end of 32nd day of heavy metal exposure, the fish from holding tanks in group V were further subdivided into group VI, VII and VIII respectively (n=10). Group VI fish were treated with crude powder of *Nelumbo nucifera* (500mg/kg diet), Group VII fish were fed with crude powder of *Aegle marmelos* (500mg/kg diet) and Group VIII fish were treated with refined standard silymarin (100mg/kg diet). The herbal drugs were mixed with fish feed and fed at a rate of 3% of body weight twice daily for 30 days.

Biochemical analysis

At the end of treatment period fresh blood samples were collected by cardiac puncture method to collect the plasma samples for vitamin C assay. The treated and control fish (n=10) were weighed and sacrificed and later dissected to isolate the whole liver and kidney tissues. They were washed with chilled physiological saline, blotted dry and weighed to prepare tissue homogenate. The post-mitochondrial fraction from the pooled liver and kidney samples were washed in ice cold 1.15% KCL solution and homogenized in four volumes of homogenizing buffer (50mM Tris-HCL mixed with 1.15% KCL and the pH adjusted to 7.4) using a Teflon centrifuge at 16,000g for 15 min at 0–4°C using Beckman L5–50B centrifuge. The supernatant was decanted and stored at -20°C until analysis. The level of thiobarbituric acid reactive substances (TBARS) was determined as per the experimental protocols outlined in the literature (Nichans *et al.*, 1968) and reported as µg/gm wet tissue. Superoxide dismutase (SOD) was estimated as per the methods of (Beauchamp and Fridovich 1971) reported as Units/mg protein. Catalase (CAT) were reported as µmoles of H₂O₂ metabolized/mg protein /min (Chance and Machly, 1955). Glutathione peroxidase (GPx) was reported as µmol/min/mg protein (Paglia and Valentine, 1967) and Glutathione-S-transferase (GST) was evaluated as per the method of Habig (Habig *et al.*, 1974) and reported as µmole of thioether formed/mg protein/min. All the reagents used for analysis were purchased from Himedia Chemicals of Mumbai, India.

Total protein estimation

The total protein content was analyzed by adopting universally accepted standard method (Lowry *et al.*, 1951) using bovine serum albumin as standard. Vitamin E in liver tissues was estimated (Barker and Frank, 1980) and the units are expressed in µg/gm of tissue. Plasma vitamin C was analyzed (Harris and Ray, 1935) and the units are expressed in mg/dL.

Statistical analysis

All measurements were performed in triplicate. The data are given as mean±SD. The comparison of the control, experimental and drug-treated groups was statistically analyzed by Student's t-test and the validity of investigation was expressed as probability (p) values. values of p<0.01 were considered significant and p<0.001 as highly significant.

RESULTS

Production of malondialdehyde (MDA)/TBARS in carp liver and kidney homogenates (Table 1 [Supplementary data]) was induced by the presence of 5ppm

concentration of combined heavy metals (p<0.001). There was a positive and strong relationship by influencing highly significant increase (p<0.001) in the elevated TBARS in both liver (2.54±0.03) and kidney (3.12±0.004) tissues compared to the control (1.51±0.04). Fish which received a supplement of *Nelumbo nucifera* (500mg/kg diet) after 30 days as a treatment period show a highly significant decrease (p<0.001) in TBARS (1.49±0.03) compared to the heavy metals exposed fish. Treatment with *Aegle marmelos* (500mg/kg diet) (a potent free radical scavenger) shows a highly significant decrease in kidney TBARS (p<0.001) compared to the heavy metals exposed fish. The standard silymarin shows similar effects as the herbal drugs. *Nelumbo nucifera* and *Aegle marmelos* inhibit the extent of TBARS/MDA production. The cytoprotective effect of herbals for holding the membrane integrity was found to be highly significant (p<0.001) compared to control and standard groups.

Fig. 1 [Supplementary data] shows a significant decrease of superoxide dismutase (p<0.001) in liver and in kidney (p<0.01) by the 32nd day of carp exposed to heavy metals. The decrease SOD in liver and kidney tissue shows the production of aggressive free radicals induced by heavy metals. Treatment with *Nelumbo nucifera* shows significant increases (p<0.001) in liver and in kidney tissues (p<0.01) compared to heavy metal exposed groups. *Aegle marmelos* is an effective agent against superoxides increasing the SOD level in both liver (p<0.001) and kidney tissues (p<0.01) compared to heavy metal exposed fish. *Nelumbo nucifera* and *Aegle marmelos* are significantly effective in increasing the SOD activity compared to the standard silymarin treated groups (p<0.001) and control fish.

Fig. 2 [Supplementary data] summarizes the decreased level of catalase in liver (p<0.01) and Kidney (p<0.01) of carp exposed to heavy metals for a period of 32 days. Treatment with *Nelumbo nucifera* show significant increase (p<0.01) in liver and kidney (p<0.01) tissues compared to the heavy metals exposed groups. *Aegle marmelos* exhibits highly significant reduction in the liver (p<0.001) and kidney (p<0.001) tissues compared to the heavy metals intoxicated fish. *Aegle marmelos* stimulates the gene expression in increasing high catalase activity compared to *Nelumbo nucifera* treated fish. The total effect of *Nelumbo nucifera* and *Aegle marmelos* increased the catalase to mitigate the toxicity and render protection evidenced by the recorded values compared to the control group and silymarin (p<0.001) treated fish.

The level of glutathione peroxidase Fig. 3 [Supplementary data] shows a highly significant decrease (p<0.001) in the liver and kidney (p<0.01) tissues respectively during the 32nd day of heavy metals exposed to *Cyprinus carpio* L. The result suggests that liver

glutathione peroxidase scavenge the H_2O_2 at a faster rate while the kidney GPx reflects a compensatory mechanism in mitigating the toxicity induced by heavy metals. Subsequent treatment with *Nelumbo nucifera* show a highly significant increase ($p < 0.001$) in liver and kidney ($p < 0.01$) compared to metal exposed groups. *Aegle marmelos* exhibit significant increase ($p < 0.001$) in liver and kidney ($p < 0.01$) compared to heavy metals exposed carp. *Nelumbo nucifera* and *Aegle marmelos* proved an equivalent effect and their values are near normal compared to control fish and silymarin ($p < 0.001$).

Fig. 4 [Supplementary data] shows the decreased glutathione-S-transferase in the liver (3.285 ± 0.03) and kidney (1.445 ± 0.003) in common carp exposed to heavy metals during the 32nd day. The GST shows a highly significant decrease in liver ($p < 0.001$) and kidney ($p < 0.001$) tissue. Treatment with *Nelumbo nucifera* for 30 days increased GST activity in liver ($p < 0.001$) and kidney ($p < 0.001$) tissue. The second drug *Aegle marmelos* imparts a highly significant increase in liver ($p < 0.001$) and kidney ($p < 0.001$) tissues. *Nelumbo nucifera* and *Aegle marmelos* increased the GST activity to dispose of the epoxide intermediates generated by the reactive oxygen species (ROS). The increase in GST might be influenced by the bioactive compounds present in the herbal drugs. The values for *Nelumbo nucifera* and *Aegle marmelos* approach the commonly used standard for statistical significance ($p < 0.01$) and control groups proved their antioxidant property.

The level of vitamin C shows a highly significant reduction ($p < 0.001$) by the 32nd day of heavy metal exposure. Treatment with *Nelumbo nucifera* increased vitamin C ($p < 0.001$) that acts as protective barriers to counteract the reactive oxygen species induced by heavy metals. *Aegle marmelos* impart a highly significant increase in vitamin C ($p < 0.001$) compared to experimental fish. *Nelumbo nucifera* and *Aegle marmelos* proved significant effect compared to silymarin ($p < 0.001$) and to control groups.

The values of vitamin E in liver tissues show a highly significant reduction ($p < 0.001$) by the 32nd day of heavy metals exposure in carp. *Nelumbo nucifera* exhibit a significant increase in vitamin E ($p < 0.001$) compared to heavy metal exposed groups. The therapeutic treatment with *Aegle marmelos* marked a highly significant increase in vitamin E ($p < 0.001$) compared to experimental fish. *Aegle marmelos* proved to be an excellent stimulator of vitamin E compared to *Nelumbo nucifera* and their values are significant compared to the standard reference compound silymarin ($p < 0.001$).

DISCUSSION

Heavy metals are considered to be extremely hazardous pollutants that generate reactive oxygen species linked with lipid peroxidation on cell membranes (Sarkar *et al.*, 1998). In most of the cases metal causes hepatotoxicity, neurotoxicity and nephrotoxicity (Valko, 2005). Earlier evidence supports the hypothesis that acute (or) chronic toxicity with heavy metals mediated the generation of injurious free radicals in various tissues of aquatic organisms (Geret *et al.*, 2002; Tagliari *et al.*, 2004).

The most important consequences of free radical production are the increase in lipid peroxidation, depletion of proteins and alteration of gene expression (Stohs *et al.*, 2000). An increase malondialdehyde level rapidly ruptures the lysosomal enzymes, stimulate cellular necrosis and destruction of the parenchymal tissues. Hence, an increase in MDA could be used as marker to assess the liver and kidney damage (Arun *et al.*, 2006). Our data reported an increase in TBARS in liver and kidney tissues confirm organ damage triggered by combined heavy metals in common carp.

Subsequent treatment with *Nelumbo nucifera* (500 mg/kg diet) and *Aegle marmelos* (500 mg/kg diet) for a period of 30 days as a treatment protocol acts as principal cellular targets in sequestration of heavy metals. A massive decrease in the MDA level in the liver and kidney tissues of treated groups indicate that the protective mechanism might be due to the presence of phytochemical compounds like Aeglin and flavonoids present in the herbal drugs (Chatterjee *et al.*, 1978; Jung *et al.*, 2003) *Nelumbo nucifera* and *Aegle marmelos* which develop a synergistic effect in controlling the vulnerability to heavy metals by their decreased MDA values compared to the control group and standard silymarin shows the equivalent effect.

A marked decline in antioxidant enzymes such as SOD, CAT, GPx and GST is observed by 32nd day of heavy metal exposure. This dramatic change might be induced by oxidative damage possibly caused by generation of reactive oxygen species at an increasing rate as the number of days of exposure increases. The overproduction of reactive oxygen species mitigate a sharp decline in antioxidants and indicate that the ability of antioxidant enzymes are quite temporary and limited (Wang *et al.*, 2002a; Lijun *et al.*, 2005).

Treatment with *Nelumbo nucifera* and *Aegle marmelos* significantly increased the antioxidant enzymes SOD, CAT, GPx and GST in liver and kidney. They maintain the defensive mechanism and cellular fluidity evidenced by the increased values of antioxidant enzymes in the *Nelumbo nucifera* and *Aegle marmelos* treated fish. The result of this study is that both herbal drugs act as effective modulators in reducing toxicity by enhancing stimulation of enzymes and increasing

immunological tolerance of fish to various environmental stresses.

Vitamin C and vitamin E play an important role in fish health as free radical scavengers by inactivating various stresses (Chew, 1995). A decrease in plasma ascorbic acid and vitamin E in liver homogenates were observed by the 32nd day of exposure to heavy metals. Vitamin C controls the oxidation reactions of fatty acids (Brake, 1997). The decrease in vitamin C suggested that dehydro ascorbic acid undergoes defensive reactions during the stress induced by heavy metals. Vitamin E acts as protective agents for the cells against the ROS. It may be useful in controlling the hepatotoxic effects generated by chemicals (Tokuda *et al.*, 1995). The decreased vitamin E causes necrosis and apoptosis in carp cells that was also supported by earlier reports (Ramanathan *et al.*, 2003b).

Treatment with *Nelumbo nucifera* and *Aegle marmelos* imparts significant increase of plasma vitamin C and vitamin E in liver of *Cyprinus carpio* L. The treated fish show an increase vitamin C compared to the control groups thus convert the oxidized ascorbic acid into dehydro ascorbic acid. The increased level of vitamin E from *Nelumbo nucifera* and *Aegle marmelos* treated groups maintain the structural integrity and fluid balance in plasma membrane. The results with reference to the tissue injury in the heavy metal intoxicated fish show demonstrable recovery when they are fed with crude powder of *Nelumbo nucifera* and *Aegle marmelos* along with the feed. The values of herbal drug-treated fish are equivalent to the standard group and compared to the control group, both herbal drugs has excellent antioxidant and cytoprotective effect.

The present study suggests on the cytoprotective effect of *Nelumbo nucifera* and *Aegle marmelos* compared with standard (silymarin). The herbal drugs enhance adaptive immunity by preserving the structural and functional integrity of important immune cells. The stimulated increase in antioxidant enzymes, vitamin C and vitamin E output the therapeutic efficacy of herbals against metal toxicity. In this respect, the inclusion of specific powders of *Nelumbo nucifera* and *Aegle marmelos* as specific nutrient supplement in fish feed is supported by this study. The diet formulated by including these powders can significantly decrease the incidence of environmental stress and increase the immune competence promoting growth and survival.

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