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Amelioration of mercuric chloride induced oxidative stress by *Hygrophila auriculata* (K.Schum) Heine via modulating the oxidant - antioxidant imbalance in rat liver

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Abstract

Medicinal plants and their phytochemical constituents are considered as paramount entity to treat various human ailments. The present study was designed to investigate the effect of ethanolic extract of *Hygrophila auriculata* against mercuric chloride induced oxidative damage in rat liver. Mercuric chloride ($HgCl_2$) (1mg/kg b.w, i.p) was administered three times in a week for two weeks to induce mercury toxicity. Mercury damage to the liver was evidenced from an increase in the activities of marker enzymes such as ALT, AST, ALP and ACP in serum. The level of lipid peroxidation was increased in liver tissue with concomitant decrease in both the enzymic and non-enzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin C and vitamin E. The glutathione metabolizing enzymes such as glutathione reductase, glucose-6-phosphatase dehydrogenase and glutathione-S-transferase were also decreased. Interestingly post treatment with ethanolic extract of *Hygrophila auriculata* (100 mg/kg b.w, p.o) for 10 days after mercury induction significantly ameliorated the mercury induced oxidative damage which was evidenced by the reduction in the marker enzymes, lipid per-oxidation and increase in the status of antioxidants and enzymes of glutathione metabolism. Thus, this remarkable hepato-protective activity of ethanolic extract of the plant might be due to the antioxidant nature, which scavenges excessive free radicals that were involved in the acceleration of per-oxidation reaction and the potent membrane protective property which ultimately render protection to the liver against $HgCl_2$ induced oxidative damage.

Key words: *Hygrophila auriculata*; Mercuric chloride; Antioxidant; Lipid per-oxidation

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Introduction

Heavy metals such as aluminum, cadmium, lead, zinc and mercury affect many tissues like liver, kidney and brain etc (Huang et al. 1996). Due to industrialization and changes in the environment during the twentieth century, humans and animals are exposed to numerous chemical form of mercury. Since mercury is ubiquitous in the environment, it is nearly impossible for most humans to avoid exposure to mercury in any of its forms on a regular basis (Fitzgerald and Clarkson 1991). All forms of mercury cause toxic effects in a number of tissues and organs depending upon the level of exposure, the duration of exposure and the route of exposure (Clarkson 1972). Since mercury is being widely used in different fields such as medical, agricultural and industrial fields, its exposure cannot be avoided by humans (Sharma et al. 2002). Heavy metals are known to produce oxidative damage in the liver tissues by enhancing peroxidation of membrane lipids (Chaurasia and Kar 1997), a deleterious process solely carried out by free radicals (Halliwell and Gutteridage 1990). Many studies have investigated possible relationship between lipid peroxidation (LPO) and cellular damage in hepatic tissues under various pathological conditions (Comporti 1985). Lewis and Willis (1962) have suggested that peroxide formation may lead to oxidative destruction of thiol groups of amino acids and proteins.

Cell membranes are targets for oxidative damage produced by xenobiotics including heavy metals (Halliwell and Gutteridage 1990). Per-oxidative decomposition of membrane lipids is catastrophic for living system. Antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase are scavenges free radicals and lipid peroxides and deoxicify them (Dwivedi et al. 1984). Previous reports suggest that LPO is enhanced by disturbances such as depletion of cellular antioxidants (Tan et al. 1984).

Many antioxidant compounds, occurring naturally from plant sources, have been identified as free radical scavengers (Duh 1998). Recently, interest has increased considerably in finding naturally occurring antioxidants from feed or medicinal flora to replace synthetic antioxidants, which are being restricted, due to their adverse side effects such as carcinogenicity, hepatotoxicity and nephrotoxicity (Ito et al. 1983). Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases and also retard the lipid oxidative rancidity in food,

cosmetics and pharmaceutical materials (Kinsella et al. 1993). In search of plant as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the past few decades (Velioglu et al.1998). Among the various natural antioxidants phenolic compounds are reported to be active, quenching oxygen derived free radicals by donating hydrogen atom or an electron to the free radical (Yuting et al.1990). Also, phenolic compounds of plant materials have been shown to neutralize free radicals in various *in vitro* model systems (Zhang et al.1996).

Hygrophila articulate (K. Schum.) Heine (Syn) is a wild herb commonly found in moisture places on the banks of tanks, ditches and paddy fields throughout India and is one of the sources of Ayurvedic drug 'kokilaksha'. Aerial parts of the plant are used ethnobotanically for the treatment of body pain, jaundice and malaria, while the seeds are used for treatment of impotence and thus as aphrodisiac (Jain 1991). Aerial parts of *H. auriculata* have been reported to contain lupeol, stigmasterol and butelin while the seeds of the plant are reported to contain mainly fatty acids (Quasim and Dutta 1967). The plant has been shown to possess hypoglycemic activity in human subjects (Fernando et al.1989), hepatoprotective activity against paracetamol and thioacetamide intoxification in rats (Singh and Handa 1999) and CCl4-induced liver dysfunctions (Shailajan et al. 2005), antitumour (Mazumdar et al. 1997), anabolic and adrogenic activities (Jayatilak et al. 1976). *H. auriculata* seeds have been reported to ameliorate the activities of antioxidant enzymes such as glutathione peroxidase and catalase in hepatocarcinoma (Ahmed et al. 2001). However, there is a paucity of information regarding antioxidant properties in experimental animals using the whole part of this plant. Therefore, the present study was undertaken to investigate the effect of ethanolic extract of whole plant *H. auriculata* against mercuric chloride ($HgCl_2$)-induced toxicity in wistar albino rats in order to validate the ethnobotanical and clinical claims of the plant.

Materials and methods

Animals

Healthy male Wistar albino rats weighing 120±30g purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, were used for this study. Animals were housed in polypropylene cages and were provided certified rodent pellet diet and water *ad libitum*. They were maintained at 27°C to 37°C with 12 h light and dark cycle. All animal experiments were performed in accordance with the strict guidelines prescribed by the Institutional Animal Ethical Committee (IAEC No. 07/041/05) after getting necessary approval.

Chemical s

Mercuric chloride was purchased from Sigma Chemical Company, USA. 5-5′dithiobis-2-nitrobenzoic acid (DTNB) and 1-chloro 2, 4-dinitrobenzene (CDNB) were purchased from SISCO Research Laboratories, Chennai, India. All the other chemicals used were of analytical grade and were purchased locally

Collection of plant material

The plant is widely distributed throughout India, Srilanka, Burma, Malaysia and Nepal. The plants were collected from Red Hills, Tamil Nadu in the month of August 2004. The plant specimen was authenticated by Dr. S. Jayaraman, Plant Anatomy Research Center, Chennai, Tamilnadu, India. A voucher specimen has been deposited at the herbarium unit of the Department of Pharmacology and Environmental Toxicology, University of Madras, Taramani, Chennai.

Preparation of plant extract

The whole plant was shade dried and coarsely powdered. The powder was then extracted with ethanol (60-70˚C) using Soxhlet extractor. The extract was dried under reduced pressure using rotary flash evaporator. The percentage yield of alcoholic extract was 12% w/w. The extract was stored in refrigerator for further studies.

Experimental design

Rats were divided into 4 groups with 6 animals in each group. The experimental design was as follows: Group I rats served as controls and were treated with normal saline (0.9% NaCl). Group II rats were administered with $HgCl_2$ (1 mg/kg b.w., i.p.) three doses per week for two weeks. Group III rats were administered with $HgCl_2$ dosage as in group II and then extract of *H. auriculata* (100 mg/kg b.w, p.o) for ten days. Group IV rats were treated with *H. auriculata* extract alone. At the end of experimental period, animals were subjected to mild ether anesthesia, blood was collected from retro orbital plexus and the serum was separated by centrifugation at 3000 rpm for 15 min at 4°C. Animals were sacrificed by cervical decapitation and the liver was excised, washed in ice cold saline and blotted to dryness. A 10% homogenate of the liver tissue was prepared in Tris-Hcl buffer (0.1 M; pH 7.4), centrifuged at 1000 rpm for 10 min at 4°C to remove the cell debris. The clear supernatant was used for further biochemical assays.

Biochemical assays

Aspartate and alanine transaminases (AST and ALT) were assayed according to the method of King 1965, acid and alkaline phosphatase enzymes in serum by the method of Bergmeyer, 1963 as described by Balasubramanian et al. 1983. Lipid peroxidation (LPO) was determined in the liver tissue as described by Devasagayam 1987, superoxide dismutase (SOD) according to Marklund and Marklund 1974 and catalase (CAT) according to the method of Sinha 1972. The activity of glutathione peroxidase (GPx) was estimated by the method of Rotruck et al. 1973. The hepatic reduced glutathione (GSH) content was estimated by the method of Moron et al. 1979. Vitamin C and Vitamin E were estimated by the method of Omaye et al. 1971 and Desai 1984 respectively. The activity of glutathione metabolizing enzymes such as glutathione reductase (GR), glucose -6-Phosphatase dehydrogenase (G6PD) and glutathione -S- transferase (GST) were estimated by the method of Staal et al. 1969, Beutler 1963, Habig et al. 1974 respectively.

Statistical analysis

The data obtained was subjected to One way ANOVA and Tukey's multiple comparison test was performed using SPSS statistical package (Version 7.5). Values are expressed as mean ± S.D. Value $p< 0.05$ was considered significant.

Result

Fig 1 shows the status of serum AST, ALT, ACP and ALP in control and experimental animals. The levels of the marker enzymes such as AST and ALT were significantly increased in serum with 27.8% and 39.5% respectively due to mercuric chloride induced hepatotoxicity when compared to group I control. In addtion the ACP and ALP levels were also increased with 24.5% and 28.5% respectively in group II animals when compared with group I control animals. On the other hand, the increased activities of AST, ALT, ACP and ALP in serum of group II mercuric chloride induced hepatotoxicity were significantly decreased in group III *H. auriculata* post treated animals. However, there was no significant changes were

observed in group IV H. auriculata alone treated animals when compared to group I control animals.

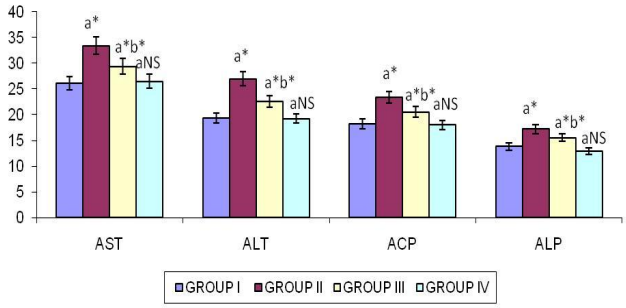

Figure 1: The levels of transaminases and phosphatases enzymes in serum of control and $HgCl_2$ treated rats. Units are expressed as: AST, ALT = µ moles of pyruvate liberated/mg protein/min. ACP, ALP = µ moles of p-nitrophenol liberated/mg protein/min. Each value represents mean ± SD of six animals, a – Group II, III & IV compared with Group I, b – Group III compared with Group II $^{*}p<0.001$; $^{\#}p<0.01$; $^{@}p<0.05$; ^{NS} – No significant

The changes in the LPO level of liver tissue of control and experimental rats were illustrated in Fig. 2. LPO was significantly increased (around 2 fold) in $HgCl_2$ treated rats (group II) when compared with control rats (group I). Whereas treatment with *H. auriculata* group II animals showed a significant fall in the LPO level.

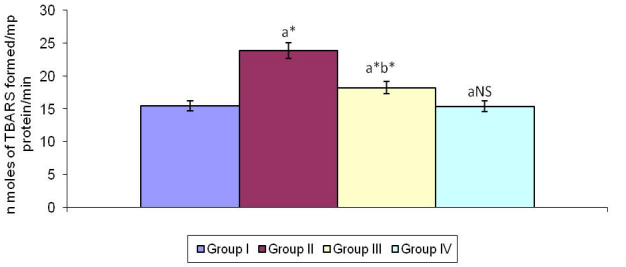

Figure 2: Effect of *H.auriculata* on lipid peroxidation in liver tissue of control and experimental rats during $HgCl_2$ induced toxicity. Each value represents mean ± SD of six animals, a – Group II, III & IV compared with Group I, b – Group III compared with Group II $^{*}p<0.001$; $^{\#}p<0.01$; $^{@}p<0.05$; ^{NS} – No significant.

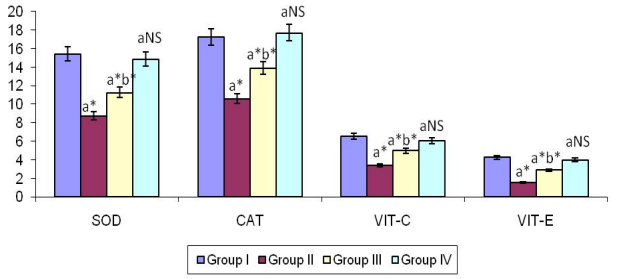

Figure 3: Effect of *H.auriculata* on enzymic and non-enzymic antioxidants in the liver of control and experimental animals. Unit are expressed as: SOD=units /mg protein: CAT=µmoles of H_2O_2 consumed /mg protein/min: GPx=µ g of GSH utilized/mg protein/min: GSH= µg/mg protein/min; VIT-E and VIT-C=mg/g of wet tissue.

The changes in the level of enzymic and non-enzymic antioxidants such as SOD, CAT, GPx, GSH, Vit-E and Vit-C in the liver of control and experimental rats were given in Fig -3. A significant decrease (2 fold) in the activity of these radical scavengers were noticed after the administration of $HgCl_2$ in group II rats, when compared with group I rats.

On the contrary, post treatment with *H. auriculata* the activities of these enzymic antioxidants were significantly reversed to normal. The activities of non-enzymic antioxidants such as GSH, Vitamin C and Vitamin E were significantly decreased (2 fold) in group II rats treated with mercuric chloride, when compared with group I control rats, which were significantly reverted to normal level in group III *H. auriculata* post treated animals (Fig 4).

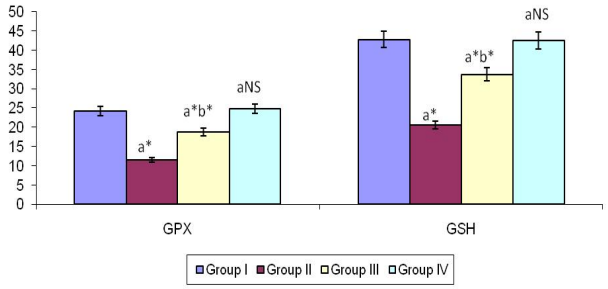

Figure 4: Each value represents mean ± SD of six animals, a – Group II, III & IV compared with Group I, b – Group III compared with Group II $^{*}p<0.001$; $^{\#}p<0.01$; $^{@}p<0.05$; ^{NS} – No significant.

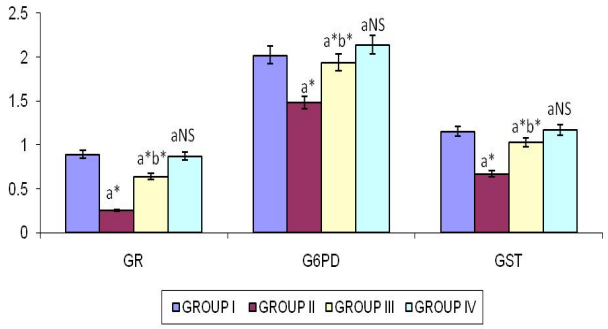

Figure 5: Effect of *H.auriculata* on glutathione metabolizing enzymes in liver of control and $HgCl_2$ treated rats. Enzyme activities are expressed as : GR - nmoles of NADPH oxidized /min/mg protein ; G6PD - nmoles of NADPH formed /min/mg protein ; GST - nmoles of CDNB - GSH conjugate formed /min/mg protein. Each value represents mean ± SD of six animals, a – Group II, III & IV compared with Group I, b – Group III compared with Group II $^{*}p<0.001$; $^{\#}p<0.01$; $^{@}p<0.05$; ^{NS} – No significant

The levels of glutathione metabolizing enzymes such as GR, G6PD and GST were depicted in Fig 5. The levels of members of the glutathione metabolizing enzymes were significantly ($p < 0.05$) decreased in group II toxicity bearing animals when compared to group I control animals, which were reverted to normal level on post treatment with *H. auriculata* and were comparable to group II animals.

Discussion

Redox disturbances are known to negatively impact the biological system through generation of ROS, which modify proteins, lipids and DNA (Choudiere, 1994). Liver being one of the target organs for mercury accumulation has witnessed the toxic insult of mercury by way of alterations in the activities of transaminases and phosphatases. Transaminases (ALT and AST) being an important class of enzymes linking carbohydrate and amino acid metabolism, have established a relationship between the intermediates of the citric acid cycle. These enzymes are regarded as markers of liver injury since; liver is the major site of metabolism (Liss et al. 1965). Phosphatases (ALP and ACP) are membrane bound and their alterations are likely to affect the membrane permeability and

produce derangement in transport of metabolites. Moreover, Plaa et al. 1986 have reported that these phosphatase enzymes act as an indicator of cholestatic changes. In this study, $HgCl_2$ administration to rats lead to marked elevation in the level of serum transaminases and phosphatases which is indicative of hepatocellular damage. This might be due to the possible release of these enzymes from the cytoplasm, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage. Several researchers have reported that increased serum phosphatases and transaminases during mercury poisoning (Sharma et al. 2005) which clearly corroborates our study. The reason for the activities of marker enzymes during the toxicity might be due to the fact that oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O_2, H_2O_2, and OH) exceeds beyond the antioxidant capability of the cell (Winterbourn 1993).Treatment with the ethanolic extract of *H. auriculata* reduced the level of above mentioned markers in serum of $HgCl_2$ induced rats. This indicates that *H.auriculata* tends to prevent liver damage by maintaining in the integrity of plasma membrane, thereby suppress the leakage of enzymes through membranes, exhibiting hepatoprotective activity. It is reported that the products of LPO inactivate cell constituents by oxidation or cause oxidative stress through undergoing radical chain reaction which ultimately leading to loss of membrane integrity (Sorg et al. 1998). The stimulation of LPO observed as result of Hg (II) administration could be due to the formation of free radicals thought an exhaustion of antioxidants leading to oxidative stress (Sanders et al. 1996). It has been reported that Hg (II) can inactivate a number of enzymes by blocking the functional sites through binding to sulphydryl groups, which are part of catalytic or binding domains (Fridovich, 1986). Thus it was suggested that in addition to depletion of intracellular thiol pools, the oxidant pathway may be a primary mechanism of induction of the response for Hg to induce oxygen free radicals or promote formation of lipid peroxides. Free radical scavenging enzymes like SOD and CAT protect the biological systems from oxidative stress. The SOD dismutates superoxide radicals (O_2-) into hydrogen peroxide (H_2O_2) and O_2 (Murray et al. 2003). CAT further detoxifies H_2O_2 into H_2O and O_2 (Bhattacharjee et al. 2006). GPx also functions in detoxifying H_2O_2 similar to catalase. Thus, SOD, CAT and GPx act mutually and constitute the enzymic antioxidative defense mechanism against reactive oxygen species (Mahboob et al. 2001). The decrease in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the metabolism of $HgCl_2$. This is further substantiated by an elevation in the levels of lipid peroxidation. Similar reports have shown an elevation in the status of LPO in liver during $HgCl_2$ exposure (Shanmugasundaram et al. 2006) and our results are in accordance with these reports. Restoration in the levels of lipid peroxidation after administration of *H. auriculata* could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids. Our results are in line with previous studies by Chaudiere, (1994) in which they have shown that *H. auriculata* exhibits excellent antioxidant property. Therefore this property of *H. auriculata* might have resulted in the recoupment in the activities of the above antioxidant enzymes to normalcy. In the present study, we have observed that the depletion of the levels of non-enzymic antioxidants such as GSH, vitamin-C and vitamin-E due to the excessive liver damage and oxidative stress caused by $HgCl_2$. The Non-enzymic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system. GSH acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation (George 2003). Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and glutathione (Das 1994). It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals (Sies 1991). The decreased levels of these enzyme activities observed during $HgCl_2$ administration might be due to the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of $HgCl_2$. *H. auriculata* treatment effectively restored the depleted levels of these non-enzymic antioxidants caused by $HgCl_2$. *H. auriculata* has been reported to maintain the GSH homeostasis in the system as suggested by Vijayakumar et al. (2006) and this might be the reason for elevated glutathione levels observed during *H. auriculata* treatment. Increase in GSH levels in turn contributes to the recycling of other antioxidants such as vitamin-E and vitamin-C (Exner et al. 2000). It shows that *H. auriculata* maintains the levels of antioxidant vitamins C and E by maintaining GSH homeostasis, thereby protecting the cells from further oxidative stress. A significant decrease in the activities of GSH dependent enzymes, G6PD, GST and GR were observed in $HgCl_2$ treated rats, which may be due to the decreased expression of these antioxidants during hepatic damage. Further, the decreased levels of cellular GSH might have also caused a reduction in their activities as GSH is a vital co-factor for these enzymes and these observations are in accordance with the reports of Gstraunthaler et al. (1983) in which they have demonstrated that $HgCl_2$ induced hepatic injury was escorted by a substantial fall in hepatic GSH level, GR, G6PD, GPx and GST activity, which improved on administration of antioxidants. It is likely that post treatment of *H. auriculata* similarly maintains the activity of GR and GST in the liver by inhibiting lipid per-oxidation and maintaining GSH levels. This is an indicative of the potent antioxidant activity possessed by *H. auriculata*.

Conclusion

From the present study, it can be concluded that $HgCl_2$ induced toxicity involves oxidative stress in its pathophysiology. The ethanolic extract of *H. auriculata* possesses a potent free radical scavenging and antioxidant activities. The extract is capable of increasing/maintaining the levels of antioxidant molecules and antioxidant enzymes in the liver and also of protecting against oxidative damage to liver tissues. The preliminary chemical examination of ethanolic extract of *H.auriculata* extract has shown the presence of polyphenols, flavonoids, triterpenes and sterols which may be responsible for the antioxidant activity. Further studies are in progress in order to the isolation of more active compounds present in the extract.

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